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DURATION OF SYMPTOMS, CLINICAL STAGING AND SURVIVAL IN CANCER OF CERTAIN SITES

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The prognosis in cancer depends on so many factors, some still unknown, that it is seldom possible to express an authoritative opinion on the outcome in any individual case. To define and to assess the importance of different factors requires the collection of large masses of data so that subsequent tabulation and analysis may be independent of variations due to chance or individual selection, as far as possible.

Since 1945 the National Cancer Registration Scheme has been collecting records of the clinical conditions of patients when they are first seen at a hospital or clinic, their treatment and their subsequent progress. In the first year of the scheme some 23,000 records were received but by the end of 1953 the annual number of recorded cancer cases had risen to more than 60,000.

This note is concerned with cases recorded between 1945 and 1949, the clinical stage of the disease at registration and the patient's statement of the duration of symptoms prior to registration. Five year survival rates are given only for those patients registered prior to 1948. Seven sites have been selected for examination, stomach, large intestine, rectum, lung and bronchus, skin, cervix uteri and prostate. These sites in men account for more than 70 per cent and in women more than 50 per cent of all deaths ascribed to cancer.

Three clinical stages are distinguished in the following tables (*a*) early, where the growth is still confined to the organ of origin; (*b*) late, where local extension has extended beyond the organ of origin; (*c*) metastatic, where metastases in distant organs are recorded at the time of registration. In this grouping no account has been taken of the presence or absence of affected regional lymph nodes which were rarely diagnosed in early cases and formed but a small proportion of late cases.

Very few cases of epithelioma of the skin (only 68) with evidence of metastatic spread were recorded and analysis of this group has been omitted from the tables.

The average duration of symptoms has been expressed in months, by the median interval. The median is that period which is exceeded by exactly one half the observations and, where the distribution of a measurement is markedly asymmetrical, as is the case here, is a more reliable estimate of the average than the arithmetic mean. This is shown in Table I together with the percentage of

cases in each stage with a declared history of (*a*) less than two months, (*b*) more than 12 months and (*c*) more than two years.

The crude survival rates in Table II are given for both sexes together and are shown (*a*) for those with a symptomatic history of less than two months, (*b*) with histories between 2 and 6 months, (*c*) between 6 and 12 months and (*d*) those with symptoms lasting one year or more.

Duration of Symptoms

In Table I, which shows separately for each sex the median duration of the three stages of each site group, it is noticeable that women tend to report a longer duration of symptoms than men. Except in the case of the early stages of cancer of skin and rectum the difference between the median values is small, considerably less than one month, but it is almost invariably present. It is also noteworthy

TABLE I.—*Average Duration of Symptoms (Median) and Percentage of Patients with Very Long and Very Short Histories* (i.e.) *of*; (a) *less than Two Months*, (b) *more than Twelve Months*, (c) *more than Two Years.*

	Males.					Females.				
			More than					More than		
Staging.	Median in months.	Under 2 months (%).	12 months (%).	24 months* (%).	Number of cases.	Median in months.	Under 2 months (%).	12 months (%).	24 months (%).	Number of cases.
Stomach										
Early	6·5	8	33	19	936	6·6	8	28	15	483
Late	5·5	13	24	12	3243	5·8	13	26	12	1828
Metastatic	4·8	14	21	11	2113	4·7	15	21	10	1270
Intestine										
Early	3·6	32	17	6	1115	3·9	29	18	7	1358
Late	3·7	31	17	7	1740	3·9	30	20	7	1783
Metastatic	3·9	25	18	7	957	4·5	21	18	7	977
Rectum										
Early	5·3	14	23	9	1941	6·4	11	26	9	1209
Late	5·7	15	25	9	2629	6·5	13	29	11	1534
Metastatic	4·9	14	23	9	912	5·6	13	25	9	547
Lung and Bronchus										
Early	5·9	6	22	10	1455	6·3	8	28	9	149
Late	5·6	10	20	9	7341	5·8	9	20	8	1071
Metastatic	4·5	14	15	6	2749	4·9	14	13	5	443
Epithelioma of the Skin										
Early	6·0	14	36	22	4666	7·5	14	41	28	2391
Late	13·2	4	52	38	901	12·2	4	66	50	549
Cervix Uteri										
Early	—	—	—	—	—	5·1	12	20	7	5491
Late	—	—	—	—	—	6·4	9	25	9	5226
Metastatic	—	—	—	—	—	6·0	11	26	10	901
Prostate										
Early	5·9	24	35	20	1102	—	—	—	—	—
Late	5·5	22	30	16	1342	—	—	—	—	—
Metastatic	5·6	14	26	10	746	—	—	—	—	—

* Included also in the previous column.

that with the exception of cancer of the large intestine and the late cases of epithelioma of the skin, the median varies little from site to site but is commonly of about six months' duration.

Using the median interval as the criterion, these seven sites fall into three broad groups.

I. Where the median duration of symptoms among those with metastatic spread is at least a month shorter than among those whose growth is still in the early stage, the late stages having an intermediate duration.

II. Where the median durations for the three stages differ by less than a month.

III. Where the median duration is shortest in the early stage and longer in the late or metastatic stage.

I.—In cancer of the stomach and cancer of the lung and bronchus in both sexes, the median duration of symptoms decreases with the increasing extent of the lesion. In cancer of the stomach the average duration among early cases is 1·7 months (males) and 1·9 months (females) longer than among those with overt metastases, the average duration of the late cases without metastases being intermediate. In cancer of the lung and bronchus the differences are 1·4 months in both cases. Also there is in the metastatic group, at both sites, a larger proportion of cases with a very short history and a smaller proportion with a long history than in either of the two other groups.

For cancer at each of these sites the proportion of cases seen in the early stage is small (stomach 14 per cent and lung 12 per cent) while the 5 year survival rates are very low.

II.—Among persons of both sexes with cancer of the intestine and among men with rectal and prostatic cancer the median durations for the different stages differ by considerably less than a month. In intestinal cancer the proportion of cases with a history of under two months is less among those with metastases than among those in the other stages but the proportions of cases with a very long history differ very little.

The median duration of cancer of the male rectum and the proportion of cases with short or long histories vary little from stage to stage, while in prostatic cancer both the proportions of cases with very long or very short histories are smaller in the metastatic group than among those with early or late growths.

In cancer of the female rectum the median duration amongst those with metastases is nearly a month less than amongst those in the early and late stage but the proportion of cases with very long and very short histories is not markedly different among those in the early and metastatic stages.

At all these sites, more cases are seen in the early stage than with metastases. Moreover the 5 year survival rates are very much higher than among the lung and stomach cases.

III.—In cancer of the cervix uteri and cancer of the skin the median duration is considerably less among early than among late or metastatic cases and the proportion of metastatic cases seen at either site is very small. In cancer of the cervix there is little difference between the proportions of those with a very short history whether seen in the early or metastatic stage though the metastatic group contains a greater proportion of cases with long histories. The 5 year

survival rate for cancer of the cervix is higher than for any of the five sites already considered.

Survival Rates

In Table II the 5 year crude survival rates of early cases receiving radical treatment are given in the first line of each section. They consist of cases where the extent of the growth was limited and where it was hoped that treatment might completely cure the condition. The survival rates vary according to the length of symptomatic history but in a different manner at different sites. In cancer of the stomach, intestine and rectum survival rates are better the longer the symptomatic history, it is only among the early epithelioma of the skin that the survival rate is higher when the history is short. In prostatic cancer those cases with a short or long history (less than two months or more than one year) have higher survival rates than those of intermediate duration. In cervical cancer the survival rate appears to be almost independent of the length of history and in cancer of the lung and bronchus though no distinct pattern is seen the survival rate of cancer treated within six months of the onset of symptoms is higher than those treated later.

TABLE II.—*Crude (5 year) Survival Rates of Early and Late Cases receiving Radical Treatment and of All Cases whether Treated or not, according to the Duration of Symptoms, expressed as percentages of Cases Registered.*

Stage.	Duration of symptoms in months. 0.	2.	6.	12+.	No. of cases.
Stomach					
Early	14·3	24·0	23·1	32·1	485
Late	8·3	6·7	8·2	17·2	392
All	2·2	3·7	3·8	7·6	4724
Intestine					
Early	36·7	35·7	38·7	47·0	993
Late	25·2	21·9	26·5	25·7	442
All	14·8	13·7	15·2	15·6	3893
Rectum					
Early	33·6	38·6	42·1	43·8	1197
Late	19·0	17·7	25·6	24·3	524
All	10·5	15·3	16·6	16·8	4324
Lung and Bronchus					
Early	12·5	16·2	8·1	10·4	411
Late	—	2·0	3·8	4·0	622
All	0·7	1·8	2·1	2·2	5760
Cervix uteri					
Early	46·8	46·6	44·3	46·9	2720
Late	28·5	27·2	29·4	29·2	1979
All	35·5	33·5	30·1	31·0	6024
Prostate					
Early	41·5	26·6	23·7	30·4	450
Late	12·5	16·5	13·7	15·6	450
All	22·1	16·1	10·9	19·6	1481
Skin					
Early	78·5	67·9	60·5	61·1	3210
Late	58·8*	42·7*	28·6*	41·4	527

* Based on less than 50 cases.

Among those cases where local extension has occurred but who are still suitable for a radical form of treatment there is rarely a regular change in survival rates with increasing length of history but with the exception of epithelioma of the skin and carcinoma of the prostate the crude survival rate of those with a history of more than six months exceeds the rate for those with shorter duration.

The crude survival rates of all cases whether treated or not are shown in the third line of each section of Table II. Only in cancer of the prostate and cancer of the cervix are the survival rates of cases with a history of less than six months higher than the rate for those with a longer history.

DISCUSSION

At no site do patients who present themselves with evidence of metastatic spread give a symptomatic history strikingly longer than those who were registered before metastases had appeared. In the case of cancers of the stomach and lung and bronchus, in both sexes, the median durations are considerably shorter among those with overt metastases than amongst those without. In cancer of the rectum the differences are smaller ; only among cancers of the cervix uteri does the median duration of those with metastatic spread exceed that of patients first seen in an early stage.

The history of the disease is dated from the appearance of a symptom—indigestion, abdominal pain, haemorrhage, retention of urine, or cough—which is thought to be an indication that the growth has already grown sufficiently to interfere with the normal function of the body. It is of course no indication of how long the cancerous process has preceded the symptom nor how large the growth has become before the symptom was caused, but it is the only measure of duration available.

To consider first the group of gastric cancers : the median duration is considerably less among the metastatic group than among those in the early stage while the symptomatic duration of those first seen in a late stage is intermediate. Not only is the average symptomatic duration of the disease shorter as the severity of the condition on registration increases but in each stage the survival rate is highest when the history is longest. If the symptomatic history is long and the growth still in an early stage, the intrinsic malignancy of that particular growth must be low compared with that in a patient with a shorter symptomatic history who already shows signs of metastastic spread. Thus it seems certain that if the degree of malignancy as estimated by the rate of growth and the tendency to invasion and metastatic spread could be computed in each case, a larger proportion of cancers of low malignancy would be found among those who present themselves in an early stage of the disease even when the symptomatic history is long, than among those not seen until the disease has progressed further. The coincidence of a longer history with a better survival rate, seen also in the early cases of cancer of the intestine and rectum, suggests some process of weeding out has occurred, leaving in the early stage only those growths of low intrinsic malignancy which have a better prospect of long survival.

A similar correlation between a short symptomatic history and an advanced stage of growth is seen also in the lung and bronchus series. In cancer of the large intestine, rectum and prostate no significant correlation either way can be discerned, while the average duration of cases of cervical cancer seen in the early stage is less than amongst those not seen until the late and metastatic stage.

Among patients with epithelioma of the skin the median duration in late cases was in men twice and in women more than one and one half as great as in early cases. Here we are dealing with a growth that can actually be seen, even in its very early stages, is in general of low intrinsic malignancy, has little tendency to produce lymphatic or distant metastases and invades adjoining structures with comparative reluctance.

Among prostatic cancers although the early cases seen within two months of the onset of symptoms have a much better prognosis than those seen later, the difference in survival between early or late cases, whether the history is under or over six months, is negligible. In the large series of cancer of the cervix uteri the survival rates vary very little within the early group while the late cases have a slightly better prognosis where the history exceeds six months.

The conclusions to be drawn from a study of these data are mainly negative in their implications but may be expressed in three propositions.

I. In cancer of the internal organs positive correlation between the length of symptomatic history and the stage of the growth is infrequent. Advanced growths with distant metastases, especially among the more rapidly lethal cancers, are frequently associated with a short duration of symptoms and a long history is equally common when a growth is still localised.

II. Prognosis as measured by the five year survival rate is in the case of internal cancer almost entirely determined by the stage the growth has reached, and it is relatively independent of the duration of symptoms, though a better prognosis is often associated with a longer history.

III. Epithelioma of the skin, where the very early stages of the growth can be visually detected, on the contrary shows a definite correlation between a short history and an early stage, a shorter history being more frequently associated with a better prognosis.

The observations presented above are not new ; Harnett (1953) reviewed the literature and on a basis of the data collected in the ' Survey of Cancer in London ' (Harnett, 1952) between 1936 and 1939, made a similar analysis for a much greater variety of sites with very similar results. He concluded that " the rate of growth of the tumour is the most important factor influencing the survival rate after radical treatment, leading to relatively long survival in spite of delay in commencing treatment." It seems also certain that the intrinsic malignancy of a growth is an important factor in determining the attitude of the patient to the need for seeking medical advice and to the resulting duration of symptoms before diagnosis. This appears true at least for growths of high malignancy and where the growth as such, is not apparent in its early stages. A change that occurs rapidly can be expected to arouse alarm at an earlier date than one which proceeds more slowly, and where recognition of the basic cause is not obvious the condition or extent of the growth when diagnosed may be the best indication of its intrinsic malignancy. Lees and Lees (1950) concluded that " It is probable that more rapidly growing and intrinsically more ' malignant ' tumours are on the average more extensive at the time of diagnosis " and Bloom (1950) writing about the prognosis of breast carcinoma suggested that " the more virulent tumours tend to present sooner than the ones of lower malignancy ". Thus a long

symptomatic history in a newly diagnosed early case of cancer may be regarded as a favourable prognostic sign, if the disease is still in an operable condition. Such a statement does not indicate that delay is not dangerous, we still know very little about degrees of malignancy and practically nothing at all as to how they may vary during the course of tumour growth. There is considerable evidence to show that an apparently indolent tumour may quite suddenly assume a rapid rate of growth while cessation of spread is not unknown as a sequel to earlier evidence of high malignancy. Conclusions arrived at from the examination of large numbers of cases are valid only for the average case, which is purely a statistical conception, having little counterpart in clinical practice where each case differs to a greater or less degree from the next. The findings of this paper do not disprove the view that early diagnosis and early treatment are of the utmost importance if survival rates are to be improved.

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BRAIN METASTASES FROM PRIMARY BRONCHIAL CARCINOMA: A STATISTICAL STUDY OF 741 NECROPSIES

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ELSEWHERE (Galluzzi and Payne, 1955) we have dealt in detail with the sources and composition of 741 necropsies which form the basis of the present article. The more general aspects of blood-borne spread were also considered in the earlier work. Our purpose here is to consider specifically brain metastases arising from bronchial carcinoma. The reason for dealing separately with this topic is that it has been a matter of special interest for a number of years and might add useful facts to the current discussions on the proportion of deaths certified as lung cancer which is due to a real increase in the disease. Many authors in the past have remarked on the prevalence of this mode of spread and some, for example Willis (1953) and Bailey (1948) have discussed its natural history and pathology in some detail.

Sources of the material

The necropsy reports which were studied were those relating to 741 patients who died with carcinoma of the bronchus in eight London Hospitals (four teaching and four regional board hospitals) during the years 1948–52. Amongst them were 94 necropsies in which no intracranial examination was made. Full details of the sources of the material are given in Table I.

TABLE I.—*The Sources of the Data*

Hospital.	Number of necropsies on patients dying from bronchial carcinoma.	Number with full necropsy including intracranial examination.
Hammersmith Hospital	156	122
St. Mary Abbots Hospital	113	90
Middlesex Hospital	87	58
St. George's Hospital	87	84
Archway Hospital	83	82
St. Mary's Hospital, Islington	99	99
Highgate Hospital	52	51
Royal Marsden Hospital	64	61
All hospitals	741	647

The incidence of brain metastases

In dealing with the incidence of brain metastases we are obliged to exclude all those necropsies at which the brain was not examined. This is justifiable if

these exclusions represent a random sample of the whole group. It is extremely likely, however, that most of these patients had no neurological signs prior to death so that, as a group, they are likely to contain a somewhat lower proportion with brain metastases than the remainder.

In order to assess factors which may influence the tendency towards the development of brain metastases we have to compare the proportions of necropsies at which brain secondaries were noted when the material is classified in a variety of ways, for example, according to sex, age, histology and site of primary.

(i) *Differences between hospitals.*—When the whole of the material was considered it was found that the brain was surpassed only by the liver and adrenals in frequency of being involved at death by blood-borne metastases from bronchial carcinoma. In the present group 25·7 per cent of necropsies showed brain secondaries though the corresponding figures for individual hospitals ranged from 17 to 43 per cent. This may well represent a greater variation than can be accounted for by case selection alone. The upper limit of 43 per cent does, however, relate to the hospital with a special neurosurgical interest.

(ii) *Differences according to sex.*—There was no evidence of any difference between the sexes in their tendency to develop brain secondaries. The incidence figures for males and females were 25·7 and 25·5 per cent respectively.

(iii) *Variation with age.*—At first sight there would seem to be evidence of a tendency for the incidence of metastases, and of brain metastases in particular, to decrease with increasing age. This impression is examined critically in (v) below.

(iv) *Differences according to the histology of the primary tumour.*—It is well recognized that undifferentiated tumours have a marked tendency to metastasize. The figures for brain metastases in this series were : undifferentiated 30·1 per cent, adenocarcinoma 32·5 per cent and squamous celled carcinoma 10·7 per cent. Let us consider the following hypothesis ; " Whether a tumour metastasizes or not depends chiefly on its histology but if it metastasizes then the general pattern of metastasis is independent of its histology." A crude test of this hypothesis can be based on a consideration of the metastasis rates for individual organs and the histological type expressed as a proportion of the number of necropsies revealing *any metastases whatever*. If the hypothesis is true we would expect that for each organ, these conditional metastasis rates would be the same for each histology. It has been shown (Galluzzi and Payne, 1955) that these rates are not generally the same (especially in the case of the kidneys). In the case of brain, too, the conditional rate for adenocarcinomas is brought into prominence being 41·5 per cent compared with 32·5 per cent for undifferentiated tumours and 21·9 per cent for squamous celled carcinoma.

(v) *Variation with age for individual hospitals.*—We have referred in (i) above to the variation in brain metastasis rates in the individual hospitals studied. We can divide these into three groups :

(*a*) A hospital with special neurosurgical interests at which 43 per cent of necropsies on patients with bronchial carcinoma revealed brain metastases.

(*b*) Two hospitals for which, considered jointly, the corresponding figure was about 30 per cent.

(*c*) The remaining five hospitals all of which yield a substantially lower incidence.

The variation of the rates with age for these three groups is illustrated in Fig. 1. It will be seen that the hospital with a special neurosurgical interest shows a particularly high rate in the fifties and the rate of 30 per cent for the second group remains virtually constant with age. Only in the third group is there any indication of a decline in incidence with age.

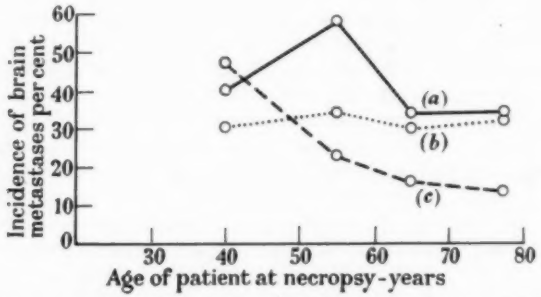

FIG. 1.—Age incidence of brain metastases by hospitals. (*a*) One hospital with neurosurgical interests. (*b*) Two hospitals showing no decline with age. (*c*) Five other hospitals.

This situation is obviously one in which we will be inclined to accept the greater values of a series of proportions as being nearer to a "true" value than the average of the series of proportions always provided these proportions are based on numbers large enough to render chance variations unimportant. We are therefore inclined to suggest that brain metastases from bronchial carcinoma occur at death in about 30 per cent of cases and that it is questionable whether this proportion decreases with age.

(vi) *Mechanical Factors.*—As has been pointed out elsewhere (Galluzzi and Payne, 1955) the high incidence of brain metastases in relation to metastases elsewhere could be explained in terms of mass of tissue alone. There are however exceptions to this simple hypothesis for example the skin, a large organ with a low metastasis rate, and the adrenals, small organs with high rates.

Some statistics from the literature

There are two quite distinct ways in which the stastistics on this subject have been presented in the literature. The first has been simply to record the proportion of necropsies on lung cancer patients in which brain metastases were found. We have seen in the last section that this proportion is, in this investigation, 25·7 per cent, but that the true figure may be nearer 30 per cent. Table II shows the extent to which the estimate of this proportion has varied in 40 years of literature on the subject. It will be seen that although the more recent investigations show, in general, a higher proportion of brain metastases, some in the early part of the century (e.g. that of Dosquet, 1921) record more than 30 per cent and some of the later ones (e.g. that of Bryson and Spencer (1951) which is by far the largest) record as low as 17 per cent.

The second type of statistic which has claimed attention is a statement of the proportion of brain metastases found to have arisen from primary bronchial

TABLE II.—*Relative Frequency of Occurrence of Brain Metastases from Primary Carcinoma of the Lung as found at Post-mortem by Various Authors.*

Author.	Periods covered by examinations.	Number of full necropsies reviewed.	Number in which brain metastases were found.	Per cent in which brain metastases were found.
Seyfarth (1924)	1900–1924	307	32	10
Olson (1935)	1900–1934	22	—	36
Wegelin (1942)	1900–1940	117	18	15
Mielecki (1913)	1906–1912	17	6	35
Dosquet (1921)	1907–1920	105	33	31
Levy Simpson (1929)	1907–1925	139	19	14
Rau (1921)	1909–1919	30	4	13
Klotz (1927)	1910–1927	24	3	13
Barron (1922)	1912–1921	13	2	15
Tuttle & Womack (1934)	1915–1933	30	7	23
Grove & Kramer (1926)	1917–1924	21	5	24
Brunner (1936)	1920–1933	99	28	28
Wustner (1941)	1920–1939	189	48	25
Dissman (1932)	1925–1931	80	13	16
Koletsky (1938)	1927–1937	100	22	22
Jaffe (1935)	1928–1933	77	19	25
Rogers (1932)	1929–1930	50	—	20
Meyer & Reah (1953)	1934–1950	303	77	25
Bryson & Spencer (1951)	1936–1947	866	—	17
Knorr (1949)	1946–1947	60	—	17 (inc. spinal cord)
Halpert *et al.* (1954)	1949–1953	92	30	33

carcinomas. There are several factors which might cause this proportion to vary chief among which are ;

(*a*) The relative proportion of deaths from lung cancer as compared with other cancers.
(*b*) The proportion of lung cancer deaths which come to necropsy.
(*c*) The proportion of cases of lung cancer giving rise to brain metastases.

Table III reviews the findings of various authors who have considered this problem. The absence of brain metastases from primary bronchial tumours in the investigation of Krasting (1906) is noteworthy, indeed the author himself remarks

TABLE III.—*Reported Frequency of Brain Metastases from Primary Carcinoma of the Lung*

Author.	Dates of examinations.	Number of histologically verified cases of brain metastases.	Percentage of those with brain metastases who had primary carcinoma of the bronchus.
Krasting (1906)	1871–1905	53	0·0
Grant (1926)	1913–1926	26	23·0
Elkington (1936)	1918–1933	72	33·3
Courville (1950)	1918–1948	221	39·36
Brunner (1936)	1920–1933	74	37·8
Rupp (1948)	1928–1948	42	50·0
Berglund & Raaf (1950)	1937–1949	36	25·0
Lenshoeck (1950)	1939–1948	20	45·0
Heppner (1952)	1947–1951	121	39·5

on this finding. In a series of 1078 necropsies revealing carcinomas there were only 19 primary lung tumours compared with 309 stomach tumours, 159 uterine tumours and 101 oesophageal tumours. This figure is too low to be helpful in the assessment of the incidence of brain metastases. Similar studies made in the latter part of the nineteenth century would, however, be most interesting and should be looked for more carefully than we have been able to do. Apart from this group, despite a variation from 23 to 50 per cent between individual reports, no evidence has been found of a tendency for any change in the incidence of brain metastases due to bronchial carcinoma over the last 30 years.

Multiplicity and association of brain metastases.

In this investigation we have chosen the word "single" to describe a brain metastasis which forms one isolated continuous mass. Others we have called "multiple". Furthermore, if no secondaries were found elsewhere in the body, we have called the deposits in the brain "solitary". If, however, other metastases were found elsewhere, then we have used the word "associated". These two notions of multiplicity and association thus give rise to four categories and it is interesting to see how the 166 instances of brain metastases in the inquiry fit into these four groups.

The figures which are set out in Table IV seem to indicate some dependence between the multiplicity and association of brain secondaries. It would appear that if metastases are present elsewhere in the body in addition to the brain, then the deposits in the brain are more likely to be multiple. Little or no difference in the multiplicity and association of brain secondaries is to be found between the three histological types.

TABLE IV.—*Distribution of Brain Metastases according to Multiplicity and Association*

	Single.	Multiple.	Total.
Solitary	23 (13·9%)	18 (10·8%)	41 (24·7%)
Associated	37 (22·3%)	88 (53·0%)	125 (75·3%)
Total	60 (36·2%)	106 (63·8%)	166 (100%)

The question of association can be pursued further by considering the actual sites of the other associated metastases. We have seen that in the series as a whole the incidence of brain metastases is 25·7 per cent. It follows that if we take any sub-group of the series, say those with adrenal metastases, and consider the incidence of cerebral metastases in this sub-group, then this figure should also not depart significantly from 25·7 per cent if adrenal and cerebral metastases occur quite independently. Table V shows the results of carrying out this procedure for six organs.

All the percentages except one are greater than the comparative figure of 25·7 per cent and, of course, this merely reflects the fact that metastases at any sites tend to be associated one with another. However, this does not explain why the percentages differ so much among themselves. These differences might have some important significance or merely reflect relative lethality. Liver metastases

TABLE V.—*Association of Brain Metastases with Metastases in Other Organs*

Other organ.	Number of necropsies with metastases in other organ.	Number with brain metastases also.	Percentage with brain metastases also.
Liver	249	56	22·5
Adrenals	225	85	37·8
Kidneys	101	40	39·6
Pancreas	77	39	51
Thyroid	24	8	33
Spleen	34	9	26

and brain metastases both tend to cause death relatively quickly so that the occurrence of one may preclude the onset of the other. Hence we have a low percentage (22·5) of liver metastases associated with brain metastases. In a similar way the high percentage (51) for pancreas may be a consequence of a relatively low lethality of metastases in this organ.

The site of metastases in the brain

For this purpose we have had regadr merely to the division of the brain into the cerebrum and cerebellum and have considered only the distribution of single metastases in these two parts.

A distribution of metastases in the brain which bears no relation to the sizes of the cerebrum and cerebellum might be used to infer something as to the route of spread into the brain. However, of sixty single brain metastases, 23 (38 per cent) were located in the cerebellum, a proportion which seems at least consistent with a fairly uniform distribution.

SUMMARY

In a series of 647 full necropsies on patients with bronchial carcinoma the brain was found to be involved in 166 cases (26 per cent): it is thought that the usual proportion may be nearer 30 per cent.

Brain metastases from undifferentiated tumours and adenocarcinomas were found to be about equally frequent (30–33 per cent) but to occur relatively infrequently from squamous-celled carcinomas (10·7 per cent). The computation of similar proportions based on all necropsies revealing any metastases serves to emphasize the rate for adenocarcinomas.

Since the beginning of the century there has been a tendency for the proportions of necropsies reported which reveal brain secondaries to increase from 10–15 per cent to 25–30 per cent though there are exceptions at both ends of the time scale. A study of the proportion of brain secondaries reported which were found to have arisen from bronchial primaries showed no upwrad trend since the turn of the century.

A brain secondary appears rather more likely to be single if it is also solitary, that is, unaccompanied by secondaries elsewhere in the body.

Brain and liver secondaries tend not to be present together at death while brain and pancreas secondaries are much more likely to be concurrent. These phenomena may, however, only represent a manifestation of relative lethality.

Considering only single metastases there is found to be no significant deviation from the expected proportions of secondaries lodged in the cerebrum (62 per cent) and cerebellum (38 per cent).

This study does not add weight to the suggestion that the major proportion of the increase in death certification for bronchial carcinoma has in fact been due to a real increase in the disease.

We should like to reiterate our thanks to all pathologists referred to in our previous article on this subject, namely; Professor T. Crawford, Professor J. H. Dible, Professor R. W. Scarff, Dr. A. B. Bratton, Dr. C. C. Bryson, Dr. A. G. Signy, Dr. M. Gillespie and Dr. J. W. Whittick. We must reaffirm that, while so generously providing us with access to their records, these pathologists must in no way be held responsible for any conclusions which have been drawn.

Once again we wish to thank Professor Smithers for his many helpful suggestions and assistance in preparing the text.

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MEDULLARY CARCINOMA OF THE BREAST

A Distinctive Tumour Type with a Relatively Good Prognosis following Radical Mastectomy

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Many attempts have been made in the past to correlate prognosis with the histological appearances of carcinoma of the breast, with varying measures of success. The most promising results have been obtained by using the concept of *anaplasia*, first evolved by von Hansemann, which can be applied to the majority of breast cancers (Greenough, 1925 ; Patey and Scarff, 1928 ; Haagensen, 1933 ; Bloom, 1950).

With the exception of certain rare kinds, attempts to relate prognosis to definite structural tumour types, such as scirrhous, medullary, lobular, and so on, have proved disappointing and have been abandoned by most pathologists. However, most workers agree that the well-defined adenocarcinomas, with a regular acinar pattern, have a better prognosis as a group than less differentiated tumours (Halsted, 1907 ; Greenough, 1925 ; Gricouroff, 1948 ; Harrington, 1952). Similarly non-infiltrating duct carcinomas and Paget's disease of the nipple have a better prognosis in general than invasive tumours. There is less agreement on the significance of the so-called " colloid ", or " mucoid ", carcinomas, some placing them in the lowest grade of malignancy (Delbet and Mendaro, 1927 ; Haagensen, 1933 ; Gricouroff, 1948), while others do not consider that the presence of mucin has any prognostic significance (Stewart, 1950).

While studying the relationship between histological grading and prognosis in patients with breast cancer seen at the Middlesex Hospital it was noted that a certain characteristic tumour type, medullary or papillary in structure, although rated as being of high or average grade of malignancy had a better prognosis than others of the same histological grade. Geschickter (1945) described medullary cancer and cancer cysts as " neo-mammary " cancer, because he thought that they arose from the primitive nipple pouch, and he stated that the prognosis in this type of cancer is better than one would expect from the histological appearances. Moore and Foote (1949), from the Memorial Hospital, New York, clearly defined this type of tumour as " medullary carcinoma with lymphoid infiltrate ". They found 52 cases in 1000 consecutive radical mastectomy specimens, 43 (82·7 per cent) of which were alive and clinically free from cancer five years after operation.

Gross and Microscopic Characteristics

The gross appearances of medullary carcinoma are usually quite distinctive. It is a spherical, well-circumsrribed tumour which may sometimes appear encapsulated. It grows by expansion rather than by infiltration which is so prominent a feature of the scirrhous type of growth (Fig. 1).

On section the tumour bulges outwards from the cut surface of the breast in contradistinction to the scirrhous form which shrinks from it. The colour is

greyish-white and, in the larger specimens, yellow zones of necrosis and red or brown areas of haemorrhage are common in the centre. Occasionally the centre is cystic. The tumour tissue looks moister and more succulent than that of other types of breast carcinoma.

Microscopically the tumour has well-defined boundaries with a circumscribed growing edge, confirming the impression of expansile growth given by the gross appearance. Sometimes there appears to be a zone of compressed fibrous tissue surrounding it.

In the commonest form the tumour cells are arranged in anastomosing cords separated by a loose fibrillary connective tissue stroma (Fig. 2 and 3). The tumour cells farthest away from the stroma commonly undergo necrosis, with the result that the bands of tumour appear to surround granular eosinophilic central masses, between which and the viable cells are cancer cells in varying stages of degeneration.

Some tumours are almost wholly papillary in structure (Fig. 4), others may be composed mainly of small clumps of cells (Fig. 5) : and both these features may be seen in some parts, and solid anastomosing cords in others, of the same tumour. Shrinkage spaces occur between the cancer cells and the stroma in the ordinary paraffin-embedded sections, but in spite of this there appears to be a basement membrane separating the two. The stroma in direct contact with the basal layer of tumour cells is formed by a narrow zone of flattened spindle cells and fine collagenous fibres.

In general the tumour cells are arranged in sheets but, especially in the papillary forms, glandular acini occur. This feratue may be so pronounced as to justify the description papillary adenocarcinoma. The cells themselves may vary greatly in appearance. Most commonly they have regular spherical nuclei, 20–30 mμ. in diameter, with one or two prominent nucleoli and scanty reticular chromatin (Fig. 6), but in some tumours the cells are more pleomorphic and the nuclei more variable in their staining (Fig. 7). Cytoplasm is usually abundant, stains well with eosin, and has a finely granular structure. Squamous metaplasia is rare, but occasionally giant forms are seen.

Mitotic figures are prominent ; seldom are there less than four per high power field, and abnormal forms are common.

EXPLANATION OF PLATES

FIG. 1.—Gross specimen of breast and axillary tissue showing a well-circumscribed medullary carcinoma with haemorrhagic area caused by biopsy incision.

FIG. 2 and 3.—Medullary carcinoma of the breast showing the characteristic structure of anastomosing cords of tumour with central necrotic zones. × 50.

FIG. 4.—Medullary carcinoma with areas of papillary growth. × 50.

FIG. 5.—Medullary carcinoma showing growth in clumps and cords of cells, and absence of tumour necrosis. × 50.

FIG. 6.—High power view of the growing edge of a medullary carcinoma. × 345.

FIG. 7.—High power of a papillary area in a medullary carcinoma in which the cells are more pleomorphic than in Fig. 6. × 345.

FIG. 8.—Growing edge of a medullary carcinoma to show the pronounced plasma-cell infiltrate in the stroma. × 400.

FIG. 9 and 10.—Low power view of a primary, which appears to be arising in a cyst, and the metastasis in an axillary node, to show the similarity of pattern. × 25.

FIG. 11.—Early medullary carcinoma showing intraduct proliferation (top) and invasive areas below. × 50.

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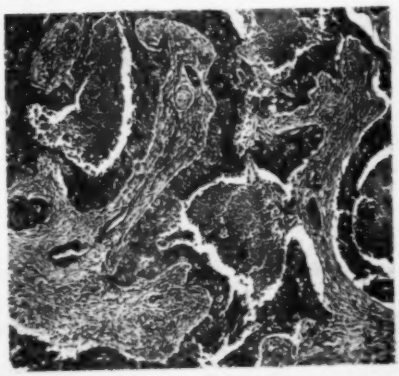

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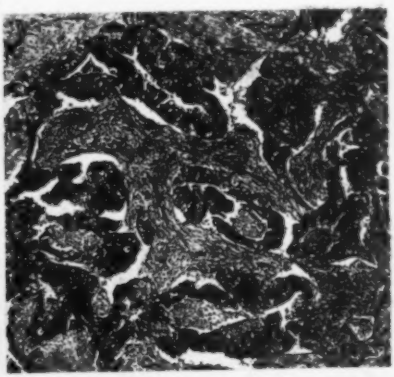

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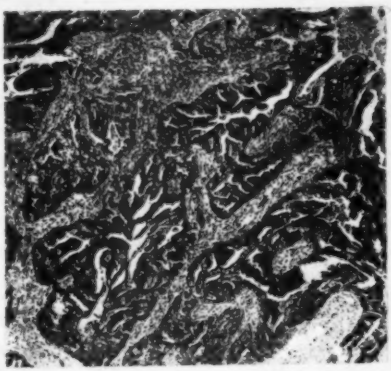

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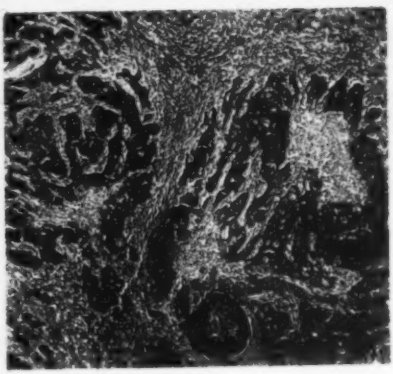

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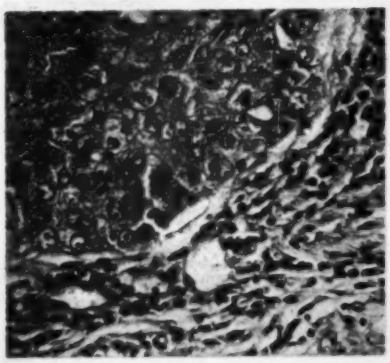

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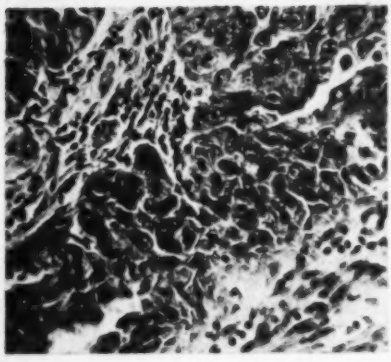

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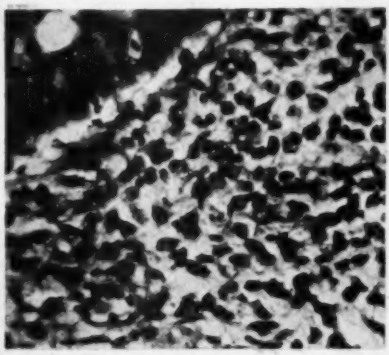

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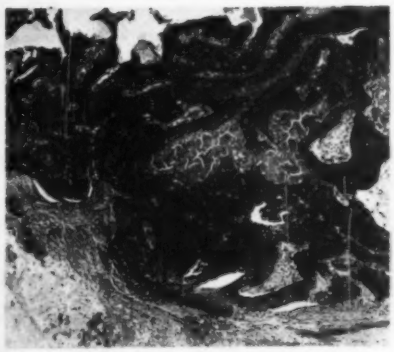

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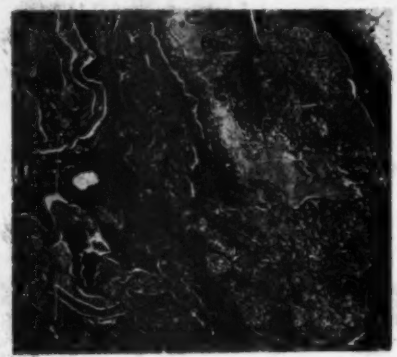

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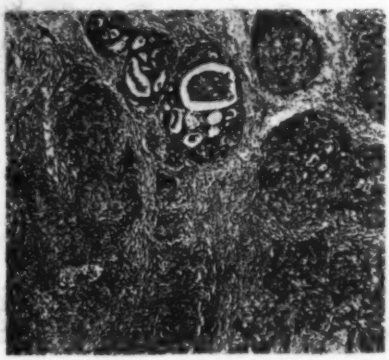

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A striking and almost constant feature of this form of carcinoma is infiltration of the supporting stroma by small round cells. This is sometimes so pronounced that the section at first sight may be taken for one of secondary carcinoma in a lymph node, and only the presence of some normal breast lobules or ducts will indicate that the tumour is in fact primary. The infiltrate is composed of plasma cells and lymphocytes in varying proportions, but commonly the former predominate. Sometimes the stroma appears to be composed almost exclusively of plasma cells and reticulum cells (Fig. 8). There does not seem to be any constant relationship between the degree of this round cell infiltration and the extent of tumour necrosis. Nor is it accounted for by ulceration and secondary infection of the skin because it occurs in tumours set deeply in the breast overlaid by intact skin.

The structure of lymph node metastases is similar to that of the primary (Fig. 9 and 10).

Material and Results of Treatment

Histological material from surgically treated cases of primary breast carcinoma in the files of the Bland-Sutton Institute of Pathology, Middlesex Hospital, from the beginning of 1936 to the end of 1950, was examined for examples of medullary carcinoma. In addition material from a large number of the patients operated upon elsewhere and referred to the Middlesex Hospital for radiotherapy between the years 1943 and 1949 inclusive, was also available.

The histological grade of malignancy of each tumour, the degree of round cell infiltration, and of tumour necrosis, were assessed before referring to the clinical notes of the patients.

	Number.	Medullary type.	Percentage.
Middlesex Hospital (1936–1950)	1224	83	7
Cases from other hospitals (1943–1949)	436	34	8
Total	1660	117	7

Of the 117 patients with medullary carcinoma 101 were treated by radical, or modified radical, operation, followed in 64 by radiotherapy to the chest wall and gland fields. In 3 patients radical operation was preceded by radiotherapy.

16 patients were treated by simple mastectomy or by local excision of the tumour, followed by radiotherapy in all but one.

1.—*Age distribution*

The youngest patient was aged 30, the oldest 76 years. In 3 patients the age was not stated in the notes.

Age.	30–39.	40–49.	50–59.	60–69.	70 or more.
Number of patients	8	38	41	22	5
Percentage distribution	7	33	36	19	5

2.—*Survivals following initial treatment*

These are presented in the following three tables.

TABLE I.—*All Treatments*

	5-year results.	10-year results.
Total number	117	60
Lost to follow-up	1	2
Post-operative deaths	2	2
Available for study	114	56
Number alive at end of stated period	88 (77%)	31 (55%)
Number alive and clinically free from cancer	82 (72%)	31 (55%)
Died of breast cancer	17 (15%)	14 (25%)
Died of other causes, no cancer clinically evident	9 (8%)	11 (20%)

TABLE II.—*Radical, or Modified Radical, Surgery (with post-operative radiotherapy in 64 patients).*

	5-year results.	10-year results.
Total number	101	50
Lost to follow-up	—	1
Post-operative deaths	2	2
Available for study	99	47
Number alive at end of stated period	81 (82%)	30 (64%)
Number alive and clinically free from cancer	77 (78%)	30 (64%)
Died of breast cancer	10 (10%)	8 (17%)
Died of other causes, no cancer clinically evident	8 (8%)	9 (19%)

TABLE III.—*Local Excision or Simple Mastectomy (with post-operative radiotherapy in 15 patients).*

	5-year results.	10-year results.
Total number	16	10
Lost to follow-up	1	1
Post-operative deaths	—	—
Available for study	15	9
Number alive at end of stated period	7 (47%)	1 (11%)
Number alive and clinically free from cancer	5 (33%)	1 (11%)
Died of breast cancer	7 (47%)	6 (67%)
Died of other causes etc.	1 (6%)	2 (22%)

Although these numbers are small it is evident that patients with medullary carcinoma of the breast, as a group, fare better than do most patients with breast cancer, and this difference is even more striking in the group treated by radical surgery. The average five-year survivals reported from most clinics in which large numbers of patients with breast cancer are treated are approximately 50 per cent following radical surgery.

The five-year survival rate of 82 per cent (78 per cent clinically free from cancer) in the radical surgery group in this series (Table II) agrees with the 83 per cent reported by Moore and Foote (1949); similarly the 10 per cent dying from breast cancer is close to their figure of 11·5 per cent.

The five- and ten-year survival rates in the group of patients treated by local excision or simple mastectomy are poor, both when compared with those of the

group treated radically, and when compared with those of any large series of breast cancers of all types. This cannot be wholly explained by selection of less favourable cases because 6 of the 15 in this group were assessed as Stage 1, 4 Stage 2, and 4 Stage 3. (One was not stated.) Even more significant is the high death rate from breast cancer in this group, 47 per cent at 5 years and 67 per cent at 10 years, compared with that of the group treated by radical surgery ; 10 per cent and 17 per cent respectively.

Influence of Stage of the Disease, Grade of Malignancy, and the Degree of Stromal Infiltration and Tumour Necrosis, on Prognosis.

1.—*Grade of malignancy*

An attempt was made to determine whether the histological grade of malignancy in the tumours of patients treated by radical, or modified radical, surgery could be correlated with prognosis in the same way as it can be for invasive breast carcinoma in general (Greenough, 1925 ; Patey and Scarff, 1928 ; Haagensen, 1933 ; Scarff and Handley, 1938 ; Bloom, 1950).

Of the 99 cases available for study (2 post-operative deaths excluded), 1 was assessed as Grade I, 30 as Grade II, and 68 as Grade III. The crude five-year survivals of patients in each grade are as follows ;

Grade.	Number.	Alive at end of 5 years.	% alive.
I (low malignancy) .	1	1	100
II (average malignancy)	30	25	83
III (high malignancy) .	68	55	81
	99	81	82

These survival figures stand in marked contrast to those published by workers who have used this method of grading in the past. For example, Patey and Scarff (1928) found 69 per cent of patients with Grade I tumours alive at the end of 5 years, 42 per cent with Grade II, and 23 per cent with Grade III, in 50 patients treated by radical operation. Haagensen (1933), who critically examined Greenough's (1925) method of grading breast cancer, found 80 per cent of his Grade I cases alive at five years, 38 per cent of Grade II, and 13 per cent of Grade III, in a series of 154 treated by radical operation : and Simmons (1933), in the discussion of Haagensen's (1933) paper, gave similar figures for 358 patients whose tumours were graded by Greenough's method.

2.—*Stage of the disease*

The stage of the disease in the patients treated by radical or modified radical surgery was assessed from the notes made at the time the patients were first seen. The Manchester system, corrected for pathological examination of the axillary lymph nodes, was used. The crude 5-year survivals of patients in each stage are as follows :

Stage.	Number and percentage.	Alive at end of 5 years.	Percentage 5-year survivals.
1	54 (55%)	48	89
2	39 (39%)	28	72
3	6 (6%)	5	83
	99 (100%)	81	82

This is quite unlike the behaviour of breast carcinoma in general for, although there is a drop in the percentage of survivors between Stage 1 and Stage 2 of 17 per cent, this is smaller than would be expected in the light of published figures. Bloom (1950), from the Middlesex Hospital, using a similar method of staging, found five-year survival rates of 75 per cent in Stage 1, 51 per cent in Stage 2, and 44 per cent in Stage 3, in 209 patients treated by radical surgery alone. Harnett (1952), in an analysis of 2152 cases of primary breast cancer in London, found a five-year survival rate of 56·5 per cent in Stage 1 and 35·1 per cent in Stage 2 ; and Harrington (1952), reporting on 7822 patients treated at the Mayo Clinic, found 78·6 per cent of Stage 1 cases alive at the end of five years, and only 32·8 per cent of those in whom the axilla was invaded.

Furthermore the percentage distribution of cases with axillary node invasion in the present series shows a reversal of the usual trend seen in large groups of cases of breast cancer, in which approximately 60 per cent of patients have pathologically proven axillary invasion at the time of initial treatment (Harrington, 1952 ; Lewison, 1955). In the present series the figure of 55 per cent of patients with no axillary metastases agrees with that of 57 per cent given by Moore and Foote (1949) in their paper on medullary carcinoma.

3.—*Degree of round cell infiltration and tumour necrosis*

In view of the almost constant infiltration of the stroma by plasma cells and lymphocytes, and the frequent occurrence of tumour necrosis in medullary carcinoma, an effort was made to determine some relationship between these factors and prognosis in the patients treated by radical, or modified radical, surgery.

The results are as follows :

Degree of plasma cell, lymphocyte infiltrate.	Number.	Number alive at end of 5 years.	Percentage of 5-year survivals.
Pronounced	51	40	78
Moderate	43	37	86
Slight	4	4	100
Absent	1	0	0

Degree of tumour necrosis.	Number.	Number alive at end of 5 years.	Percentage of 5-year survivals.
Pronounced	16	13	81
Moderate	22	18	82
Slight	29	23	79
Absent	32	27	84

No significant relationship can be established by these figures.

Tumour Size in Relation to Prognosis

The smallest tumour in this series was half an inch in diameter, the largest five inches. In 87 cases treated by radical, or modified radical, operation the size of the tumour was stated. Its relationship to prognosis is shown in the following table ;

	Up to 1 in.	1 in. + to 2 in.	2 in. + to 3 in.	More than 3 in.	Total.
Number of cases	39	38	9	1	87
Number alive at 5 years	32	31	6	1	70

There is nothing in these figures to indicate a firm relationship between tumour size and prognosis. Moore and Foote (1949) reached a similar conclusion when they analysed their 52 cases of medullary carcinoma, although in their Table III they show that in all cases of primary breast carcinoma treated by radical surgery at the Memorial Hospital, New York, a definite relationship could be shown between size and prognosis ; the larger the tumour the worse the prognosis.

Length of survival in patients dying of breast cancer

Of the total series of 117 patients 23 died of breast cancer, the shortest survival following operation being 7 months, the longest 98 months. The mean duration of life was 40·6 months. Half the number were dead 36 months after operation. This is similar to survival times published by other workers in treated patients (Scarff and Handley, 1938 ; Gricouroff, 1948).

It is interesting to note that in this series no patient with recurrences survived for 10 years : 17 of the 23 died before 5 years after operation, and only 6 (with cancer) lived longer than 5 years.

DISCUSSION

Although medullary carcinoma of the breast is recognised in America as a tumour with a relatively good prognosis following radical mastectomy (Moore and Foote, 1949 ; Stewart, 1950 ; Ackerman, 1953), this does not seem to be widely known in this country. Willis (1953) does not mention it as a pathological entity, and considers that histological subdivisions of mammary carcinoma are arbitrary and may be misleading, owing to variation in pattern in different parts of a single tumour. Some textbooks of pathology describe " medullary " or " encephaloid " carcinomas of the breast and generally consider them to have a bad prognosis (Dible and Davie, 1950 ; Muir, 1941).

That these tumours have a relatively high degree of *potential* malignancy, as indicated by the fact that most of them can be placed in Grades II or III on histological criteria, is suggested by the poor survival and high cancer death rates, in patients treated by simple mastectomy or local excision ; and also by the fact that no patient in the series who developed recurrences lived ten years from the time of operation in spite of energetic radiotherapy and hormone treatment.

The high survival rate in patients treated by radical surgery, even when the axilla was invaded, is probably partly explained by the fact that in the 36 cases in which suitable pathological material was available, 23 showed invasion by carcinoma of one node only, and in only two cases did more than three nodes contain metastases. Moore and Foote (1949) found that a significant number of their patients with medullary carcinoma had metastases confined to a single axillary lymph node.

Geschickter's (1945) views on the origin of these tumours from primitive nipple pouch structures has been mentioned above. The impression gained from study of the material in this series is that they arise from areas of intraduct proliferation, usually in many ductules at once. This is at first wholly confined to the ducts, but later extends into the surrounding stroma (Fig. 11).

Opinions differ regarding the significance of the inflammatory reaction in the stroma which is so prominent in these tumours. Ewing (1940) thought it was a sig-

nificant feature of malignant tumour growth in general, and that it must be regarded as a defensive process. He believed that well marked reaction signified a pronounced capacity to limit tumour growth, but did not necessarily mean that the effort would be successful. McCarty (1922) considered lymphocytic infiltration of the stroma about a malignant tumour to be one of the manifestations of the host's defences against carcinomas of the stomach, breast, and rectum. Greenough (1925), on the other hand, thought that a high degree of round cell infiltration was indictive of tumour necrosis and was unreliable as an indication of host resistance to cancer. Dawson and Tod (1934) concur with this view and believe that in cases in which there is lymphatic blockage by tumour cells there may be extravasation and proliferation of lymphocytes in the stroma.

In the present series no correlation was evident between the degree of tumour necrosis and the density of the plasma cell- lymphocyte infiltrate, and in 30 cases with no necrosis the degree of infiltrate was assessed as either heavy or moderate. Moore and Foote (1949) consider the infiltrate to be an intimate part of the lesion unrelated to tumour degeneration or haemorrhage, and suggest that it may be an indication of some maladjustment between tumour and host. In their opinion the favourable prognosis supports such an hypothesis.

Whether or not the relatively good prognosis of medullary carcinoma can be attributed to a lesser degree of "immunologic amnesia" (Hauschka, 1952), and the inflammatory infiltrate be a visible sign of some slight tumour antigenicity, it does appear that the individual tumour cells possess greater cohesion than those of other forms of breast carcinoma. This is suggested by their manner of growth in clumps or cords, and may explain the circumscription and reduced tendency of the tumour to form early metastases in spite of its rapid rate of growth.

Clinically one of the hazards in dealing with the early case of medullary carcinoma is that by its mobility and apparent encapsulation it may be diagnosed as a fibroadenoma. Several cases in the present series were so diagnosed and were treated by local excision. Two further cases in young women were thought to be deep-seated abscesses and were incised; only when a malignant sinus persisted was the correct diagnosis revealed.

SUMMARY AND CONCLUSIONS

The gross and microscopic features of a distinctive type of breast carcinoma—medullary carcinoma with lymphoid infiltrate—are described. Its incidence, in patients with primary breast cancer presenting for surgical treatment, is about 7 per cent.

An analysis of the subsequent history of 117 patients with this type of tumour revealed a marked difference in the five- and ten-year survival rates between those treated by radical (or modified radical) surgery, and those treated by simple mastectomy or local excision.

Of the 99 patients treated by the more radical operations 81 (82 per cent) were alive five years after operation, and 77(78 per cent) were clinically free from recurrence; 10 (10 per cent) died of breast cancer.

Only 7 (47 per cent) of 15 patients treated by local excision or simple mastectomy were alive at the end of 5 years, and of these 5 (33 per cent) were clinically free of cancer. Seven (47 per cent) died of cancer.

At the end of ten years 30 patients (64 per cent) out of 47 available for study

were alive and clinically free of cancer-in the radically treated group, and 8 (17 per cent) died of cancer.

In the group treated by local excision or simple mastectomy only one out of 9 survived ten years, and 6 had died of the disease.

In the group treated by the radical procedures no correlation could be found between the histological grade of malignancy, stage of the disease when presenting for treatment, the degree of plasma cell-lymphocyte infiltrate in the stroma, or the extent of tumour necrosis, and the subsequent clinical course.

It is suggested that the relatively favourable prognosis in patients with this type of tumour, in spite of its inherent malignancy, may be due to greater cohesion of the cancer cells with a lessened tendency to produce early metastases.

The danger of diagnosing and treating the early lesion, because of its mobility and apparent encapsulation, as a benign condition is emphasised.

I wish to thank Dr. A. C. Thackray for helpful criticisms, and the many pathologists from other hospitals who lent histological material from patients referred to the Middlesex Hospital for radiotherapy.

I should like also to thank Miss J. E. Chambers and her staff of the Follow-up department, Middlesex Hospital, for their efforts to trace patients in this study.

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ANGIOSARCOMA IN A LIMB THE SEAT OF SPONTANEOUS LYMPHOEDEMA

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IN 1948 Stewart and Treves reported six cases of lymphangiosarcoma occurring in a limb the seat of lymphoedema consequent upon radical excision of the breast for carcinoma. Since then a further ten cases have been described in all of which angiosarcoma has appeared in lymphoedematous arms of patients previously subjected to radical mastectomy for cancer of the breast (Feraro, 1950; Cruse, Fisher and Usher, 1951; Froio and Kirkland, 1952; Jessner, Zak and Rein, 1952; Vos, 1952; Rawson and Frank, 1953; Hilfinger and Eberle, 1953). In the light of these observations it seems suitable to record a case of angiosarcoma developing in a limb the seat of spontaneous lymphoedema, and in a patient free from other malignant disease.

CASE REPORT

The patient was a female who at the time of presentation in 1951 was aged 60 years.

In 1940, eleven years previously, both legs had become swollen, the swelling gradually increasing over a period of six months. At that time it was noted that the patient suffered from arterial hypertension. The swelling of the legs persisted and was not relieved by rest but the patient was otherwise well and active. Apart from the gradual increase in the swelling of the legs, no change occurred in the patient's condition until about February, 1948, when a purple discoloration appeared on the right shin, followed by purple blisters there, which broke to

EXPLANATION OF PLATES

FIG. 1.—Clinical photograph of stump August, 1951.

FIG. 2.—Coronal section of stump of right thigh showng subcutaneous haemorrage and necrotic tumour nodules.

FIG. 3.—Specimen of right lung, aorta, diaphragm and thoracic lymph nodes showing tumour deposits in pleura and diaphragm and massive haemorrhage and tumour replacement in lymph nodes.

FIG. 4.—Section of biopsy specimen of early nodule showing communicating vascular diffuse channels in the upper corium. H.E. × 145.

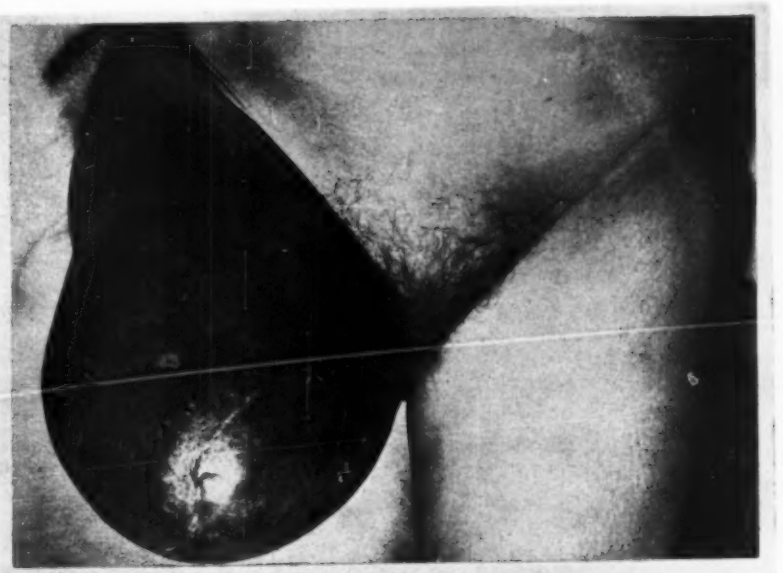
FIG. 5.—Section taken from right thigh post mortem showing much necrosis with a persistence of the vascular tumour pattern. H.E. × 90.

FIG. 6.—Section of lung showing proliferating masses of angiosarcoma destroying lung architecture and associated with haemorrhage and oedema. H.E. × 36.

FIG. 7.—Same section as Fig. 6; higher power view of angiosarcomatous area showing the crowded irregular cells lining the communicating vascular channels. H.E. × 250.

FIG. 8.—Section of tumour in lung showing the tumour cells lying for the most part within the reticulin membrane of the vascular channels. Reticulin impregnation and Carmalum. × 205.

FIG. 9.—Pulmonary lymph node showing massive replacement by tumour and haemorrhage H.E. × 36



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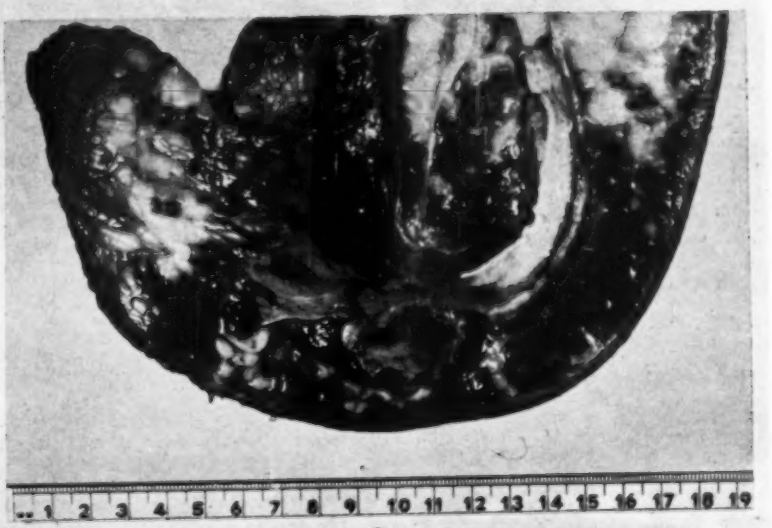

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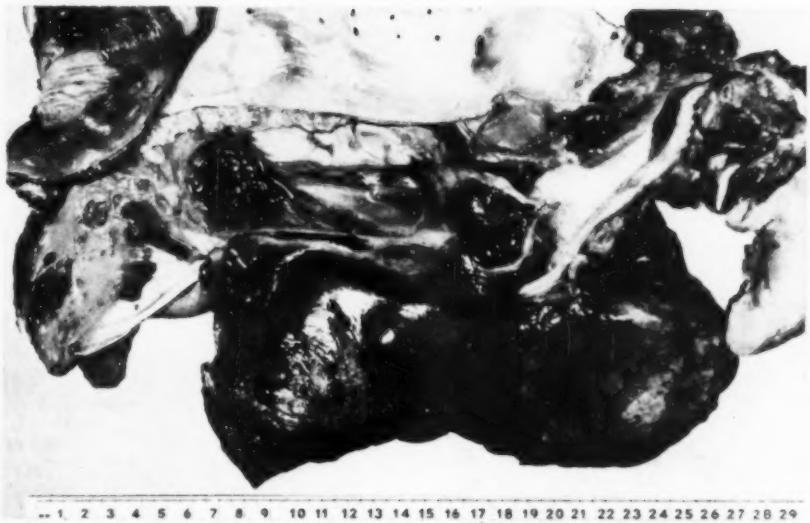

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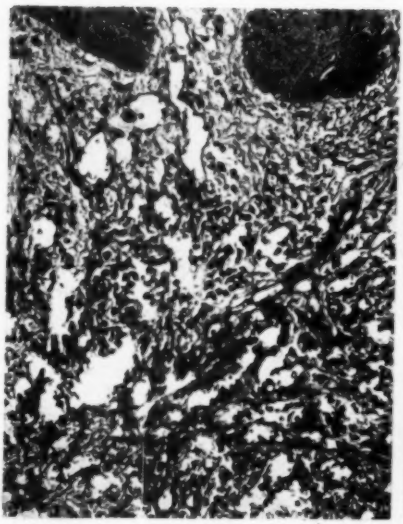

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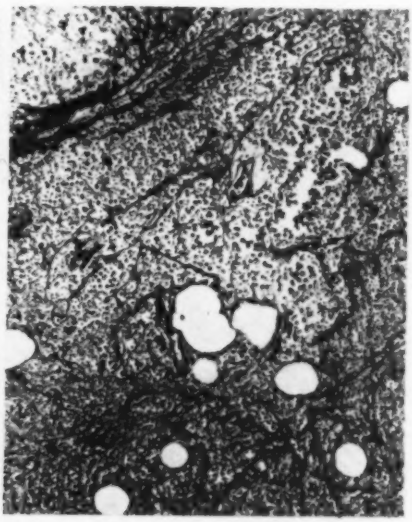

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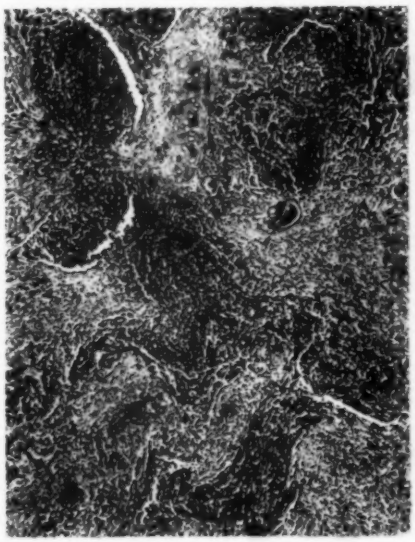

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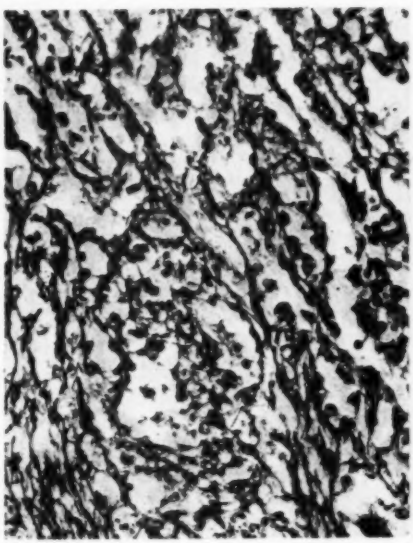

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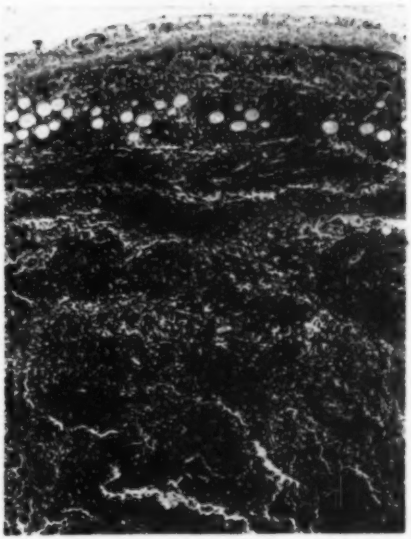

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discharge blood but no pus. This was accompanied by slight local pain but the patient could still walk easily for several miles.

On 18th May 1948 the patient presented at the Central Middlesex Hospital. There was a considerable degree of firm swelling of both legs, particularly below the knees. This swelling was solid and did not pit. On the anterior aspect of the right leg were numerous purple haemorrhagic vesicles and a few large haemorrhagic bullae. There were also two raised purple fleshy areas about 4 × 2 cm. in size. Apart from an arterial pressure of 230/160, no other abnormalities were observed.

A week's course of penicillin was given without effect. A clinical diagnosis of haemangiosarcoma was then made and this was confirmed by biopsy. The bullae shortly disappeared to leave numerous small fleshy masses.

A course of X-ray therapy was given from 11.vi.48 to 16.vii.48 with the following factors: K.V. 140. F.S.D. 25 cm. Ma. 7. Filter 0·2 mm. Cu + 1·0 mm. Al. H.V.L. 0·3 mm. Cu. The lesion was treated through two 12·5 cm. circles. Dose to the upper area was 2150 "r"; the dose to the lower area was 2750 "r". At the conclusion of treatment the lesions were smaller and paler and there was no bleeding.

Three weeks later the patient was readmitted to hospital as the discoloured area had become infected. New vesicles had developed and nodules had recurred in the treated area. On 16.viii.48 mid-thigh amputation was performed. Convalescence was uneventful.

Apart from some discomfort in the stump the patient remained well until April 1950 when she developed vaginal bleeding. A cervical polyp was removed on 21.viii.50 and at that time the swelling of the remaining (left) leg was noted to be unchanged, and there was no evidence of recurrence of the sarcoma for which the right lower limb had been removed.

In August 1951 the patient fell and struck the stump of the right lower limb against a chair. During the following fortnight the stump became swollen and painful and the patient was then readmitted to the Central Middlesex Hospital. The stump was tense, hot, blue and shiny. A haematoma of the stump was suspected but attempted aspiration only produced a very small quantity of blood. The swelling increased and pain at the end of the stump and in the right groin became very severe. The patient was admitted to Hammersmith Hospital for X-ray therapy on 18.xi.51.

At that time, on examination, the general condition was fair. The patient was a rather stout woman, still somewhat hypertensive (190/130). There was an element of anaemia (Hb. 55 per cent), and there was a marked solid, non-pitting oedema of the left foot and leg. On the right side, the mid-thigh stump was grossly swollen up to the groin, and the skin over it was tense and shiny. Purplish discoloration of the skin extended over the anterior surface of the stump as far as the inguinal ligament, and for about half this distance on the posterior surface. This discoloration of the skin gave it a curious leathery appearance (Fig. 1). There were very numerous haemorrhagic vesicles on the stump, varying in diameter from 1·0 to 5·0 mm. and between these the skin was brownish, having the appearance of an extravasation of altered blood. The whole area was extremely painful but not acutely tender. The analogy between the disease from which this patient suffered, and the syndrome recorded by Stewart and Treves (1948) was now detected.

X-ray examination showed an effusion at the base of the left lung and congestive changes in both lungs. Movement of the diaphragm was free; the left ventricle

was hypertrophic; the aortic arch was full; there was no evidence at that time of mediastinal or pulmonary metastases.

X-ray therapy was begun on 12.xi.51. By 26.xi.51 the pain was much less severe, the skin looked less tense and the discoloured area no longer reached the inguinal ligament, but the general condition had not improved. The patient complained of extreme nausea and her anaemia persisted. On 12.xii.51 treatment was discontinued. The obviously haemorrhagic vesicles had disappeared. There was now pain on the left side of the chest and the general condition was clearly deteriorating. On 18.xii.51 acute dyspnoea developed. the pain in the chest was worse and there was a small haemoptysis. Thereafter the general condition became rapidly worse and death occurred on 20.xii.51.

Postmortem findings (abridged)

The *external appearances* were those of a well nourished elderly woman. The right lower extremity had been amputated at mid-thigh and the stump was firm with a thickened oedematous and pigmented skin. The left thigh, leg and foot showed a tense brawny oedema, most marked in the foot. This limb showed no sign of tumour.

The subcutaneous tissue of the stump of the right thigh was diffusely haemorrhagic and contained five necrotic tumour nodules, the largest of which measured 3 cm., and the others 1 cm., in diameter. Muscle had been penetrated to some extent but the bone was free of tumour. The inguinal lymph nodes were not enlarged (Fig. 2).

The left inguinal region was dissected and the main ilio-femoral vessels were normal and patent. No obvious cause was found for the oedema.

Respiratory system.—The larynx and trachea appeared healthy. There was a pleural effusion to the amount of 1·5 litres in each pleural cavity. There were numerous small (2–5 mm.) deposits of tumour scattered throughout both parietal pleurae, and the lungs showed compression collapse of both lower lobes. The left lung weighed 415 g. and numerous metastatic deposits of tumour (about 2 cm. in diameter) surrounded by haemorrhagic parenchyma were seen in it, most numerous in the upper lobe. The bronchi contained dark blood. The right lung weighed 450 g. and metastases were found in its upper lobe also, some of them confluent with pleural deposits. The root nodes of the right lung and the subcarinal region were replaced by haemorrhagic tumour and a small lymph node just above the left diaphragm was also replaced by tumour. Both domes of the diaphragm were the seat of tumour deposits (Fig. 3).

The *heart* showed mild left ventricular hypertrophy and patchy fibrosis of the myocardium.

The other organs appeared to be healthy.

Microscopical findings

The first specimen excised in 1948 shows in the upper corium neoplasm which consists mainly of communicating vascular channels, most of them containing erythrocytes. There is no perivascular infiltration of cells, and no capsule can be identified. The epidermis is not involved by this process; the appearances are those of a capillary angioma probably malignant (Fig. 4).

Sections taken from the right thigh at autopsy show evidence of old and recent

haemorrhage into the fat, and necrotic tumour deposits in the subcutaneous tissue. There are no remaining viable tumour cells, but in some parts a vascular pattern had been retained (Fig. 5).

In the *lungs*, secondary deposits are present showing the structure of multiple communicating tubes with a lining of endothelial cells in excessive numbers, crowded together. In some parts, the lumina of these tubes are obliterated by endothelial cells ; the cells in these channels are in places one layer, and in places many layers thick. Reticulin silver impregnation preparations show that these crowded cells are on the inner aspect of vascular channels and are therefore in fact " endothelial ". The cells vary in diameter from about 10–20 μ and the cytoplasm is usually just a narrow eosinophilic rim. Occasional cells with relatively more cytoplasm show vacuolation, some of the vacuoles containing a red blood cell and resembling true angioblasts. The nuclei of the cells vary in size and chromasia ; they are mostly oval in shape and occasional giant forms are seen. Some of the cells are kidney-shaped. Nucleoli are not prominent. Mitotic figures are common, about 1 in every 2 or 3 high-power fields. Red blood cells lie freely in the vascular channels. The tumour deposits are not encapsulated, but there are several fibrous bands coursing through the tumour and some fibrous tissue condensation on the periphery (Fig. 6, 7, 8).

There is a variable amount of haemorrhage in the tumour masses and the surrounding lung alveoli for a distance of 2–3 cm. are filled with red blood cells. The subpleural deposits are a mixture of tumour and haemorrhage.

The *pulmonary lymph nodes* are extensively replaced by massive tumour deposits, with a considerable degree of haemorrhage and necrosis. Here the characteristics seen in other deposits are reproduced (Fig. 9).

The structure of the tumour, numerous communicating vascular channels lined by excessive numbers of endothelial cells on the inner aspect of the reticulin framework, together with the obvious malignant character of the cells seem to justify the diagnosis of angiosarcoma.

Apart from a small focus of fibrosis in the myocardium the other organs were healthy.

DISCUSSION

The six cases of lymphangiosarcoma observed by Stewart and Treves (1948) in upper limbs the seat of chronic post-mastectomy oedema were in women between 37 and 60 years of age. The shortest duration between the breast operation and the onset of the sarcoma had been six years, the longest twenty-four years. These authors were satisfied that the histological picture in each of these tumours was quite distinct from that to be expected from recurrence of carcinoma in the affected limb. It is perhaps notable that a colour photograph of one of the patients of their series show a limb in some respect similar to the stump if the patient reported here. Stewart and Treves (1948) were not able to find an example of the tumour which they reported in any form of chronic oedema other than that which follows radical mastectomy. In our case the tumour appears to be an angiosarcoma that has developed in a limb the seat of that form of chronic oedema which is usually termed " spontaneous lymphoedema ". In our case this oedema had been present for eight years before any signs of tumour were observed. This tumour is of a rare kind and its concurrence with chronic oedema, in the light of the experience of Stewart and Treves and subsequent authors, suggests some

kind of connection between the chronic oedema and the vascular tumour which subsequently developed in the oedematous limb.

It seems important to substantiate here the clinical diagnosis of " spontaneous lymphoedema " on the one hand and the pathological diagnosis of angiosarcoma on the other.

The history of the chronic oedema seems fairly characteristic of that variety which is usually called spontaneous or idiopathic, beginning in the foot and leg of one extremity and subsequently ascending in that extremity and affecting the contralateral extremity also. There was no history of deep venous thrombosis and at autopsy the veins of the remaining extremity were not found to be thrombosed. There was no history of recurrent infection. there had been no exposure to protozoal disease and autopsy showed the absence of any such tumour as might have been present eleven years before and slowly productive of a malignant oedema. The tumour which developed in the right limb was of very rapid growth and of characteristic appearance, and it seems unlikely that it could have been present and associated with malignant oedema for so long a period as eight years before overtly manifesting itself. The failure of the " solid " oedema to pit is characteristic of longstanding chronic oedema of this spontaneous variety.

So far as the pathological diagnosis is concerned, this metastasizing tumour of small vessels whose lumina are crowded with proliferating cells of mainly endothelial type, could hardly be labelled other than angiosarcoma. The tumours described by Stewart and Treves as a sequel of postoperative lymphoedema were regarded by these authors as lymphangiosarcomas. In many histological respects the tumour in our case is similar to the tumour described by these authors, but we consider that the presence of red blood cells in most of the vascular channels in our case requires a diagnosis of blood vessel tumour rather than of lymphatic vessel tumour. Hilfinger and Eberle (1953) also entertain the possibility that these tumours may be haemangiosarcomas, and the tumour reported by Cruse, Fisher and Usher (1951) as a lymphangiosarcoma, contained so much altered blood that it was confused with a melanoma until the pigment was identified by stains for free iron. Jessner, Zak and Rein (1952) were struck by the amount of associated haemorrhage and concluded that in their case there was a blood vessel as well as a lymphatic component. The relationship between haemangiosarcoma and lymphangiosarcoma is so close that we cannot entirely exclude the possiblity that the tumour which we have reported has arisen in the lymphatic endothelial cells which, coming into close relationship with adjacent blood vessels, have shown differentiation into haemangiosarcoma. The distinction between our tumour and those described by the authors cited is rather fine and it is possible that there may be an overlap between the two groups.

We have been at some pains to arrive at a distinction between the tumour here reported and Kaposi's sarcoma, for the naked eye appearances might have suggested that alternative diagnosis. Stewart and Treves (1948) and Feraro (1950) called attention to the resemblance between the tumours they reported and Kaposi's sarcoma too.

Kaposi's sarcoma, which is generally regarded as a malignant reticulo-endothelial neoplasm, occurs in the form of reddened macular areas which darken and become nodular, which may finally ulcerate, and which may ultimately ensheath the greater part of a limb by thickened discoloured skin. The whole limb is frequently oedematous.

In his original paper, Kaposi (1895) described sixteen cases of round-cell and spindle-cell sarcoma associated with capillary haemorrhage. Dörffel (1932) in a careful study of sixteen cases of Kaposi's sarcoma described in detail the microscopical features in lesions of varying duration. In the very earliest lesions he observed the presence of mild endothelial proliferation associated with a well marked perivascular infiltration of cells. Throughout the infiltration of cells he was able to demonstrate fine collagen. He considered the fully developed lesion to be characterised by masses of spindle-shaped cells which overshadowed the earlier angiomatous features of the tumour. Kaminer and Murray (1950) described a more uniform basic histological pattern in forty-one cases, corresponding closely to the fully developed lesion of Dörffel (1932)—high vascular spindle-cell tissue. McCarthy and Pack (1950), however, confirmed the essentially inflammatory early appearance of perivascular infiltration and incidentally they noted the rarity of Kaposi's sarcoma in females.

The first biopsy specimen from the case which is the subject of the present communication was obtained within three months of the appearance of the lesions, and there are no infiltrative perivascular changes present, nor is the cellular pattern that of a highly vascular spindle-celled sarcoma rich in mitoses. The thin-walled blood vessels which it presented as a basic pattern are not described by those authors who have written of Kaposi's sarcoma. The mode of appearance of the lesions was not along blood vessels, as it was in Dörffel's series, nor was it possible to demonstrate a central large blood vessel which has sometimes been regarded as characteristic of the early Kaposi lesion. " Protein bodies " such as those described by Kaminer and Murray (1950) in their cases were sought but not found.

It is said that in Kaposi's disease oedema may sometimes precede the appearance of tumour nodules. One of the patients of Kaminer and Murray (1950) had an attack of oedema lasting three weeks before the appearance of a Kaposi tumour and in another of their cases these authors were unable to decide whether mild oedema had preceded the development of a Kaposi nodule by two years, or whether the patient had small tumours at the time of the first development of oedema. Aegerter and Peale (1942) described a patient who apparently suffered from chronic lymphoedema in the lower limb, subsequently developing a small lesion which they considered to be a Kaposi's sarcoma. The illustration which accompanies the record of this case shows a capillary angioma with no perivascular infiltration and with a histological appearance not very dissimilar to that in the case we are now reporting. This case no doubt influenced Aegerter and Peale in regarding Kaposi's sarcoma as merely an angiosarcoma which happens to affect skin, a view which is at variance with most other authors. This case of theirs, the first which they reported, is perhaps more comparable to our case than to most other recorded cases of Kaposi's disease.

Histological niceties apart, there are two considerations which would seem to distinguish our case from cases of Kaposi's disease preceded by oedema. First, in the case of the right leg, the highly malignant, metastasizing and ultimately fatal vascular tumour was preceded by eight years of chronic oedema ; it is difficult to believe that the early oedema was merely the expression of a malignant procses already present but not yet overtly manifest. Secondly, the left lower limb, which at the time of death had been the seat of chronic oedema for no less than eleven years, did not at post mortem disclose any evidence of tumour at all.

It does seem inevitable that we must regard the case that we here report as one of bilateral chronic spontaneous oedema with the subsequent development of fatal metastasizing angiosarcoma in one of the affected limbs only. Angiosarcoma can, it would appear, take origin in a limb the seat of chronic oedema independently of previous neoplastic disease or operation.

Stewart and Treves (1948) concluded that the association between the oedema which follows amputation of the malignant breast on the one hand, and angiosarcoma of the arm on the other, might be explained as the effect of a general systemic carcinogen producing multiple tumours. The occurrence of angiosarcoma in a limb the seat of oedema, but in a patient free from other malignant disease, makes this hypothesis less tenable, and suggests that the relationship, and there now seem to be good grounds for assuming that such a relationship exists, is more directly between oedema and angiosarcoma, rather than between carcinoma and angiosarcoma.

SUMMARY

A description is given of a fatal case of metastasizing angiosarcoma developing in a lower limb the seat of longstanding chronic spontaneous oedema, the oedema having been bilateral but the tumour developing only on one side.

A distinction is drawn between this condition and Kaposi's disease.

This syndrome of angiosarcoma in a limb the seat of a chronic oedema due to a cause other than malignant disease or operation, would appear to have some bearing on those angiosarcomas previously reported as occurring in limbs the seat of chronic oedema, but heretofore exclusively in the oedematous arms of patients previously subject to radical mastectomy.

We should like to thank J. G. Griffin and L. J. Wright for the sections and E. V. Willmott for the photographs.

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FURTHER STUDIES ON THE PLASMA LYMPHOCYTOSIS STIMULATING FACTOR IN CHRONIC LYMPHATIC LEUKAEMIA AND SOME OTHER DISEASE STATES

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In a previous communication, Metcalf (1956) reported the presence of a lymphocytosis stimulating factor in the plasma of patients suffering from chronic lymphatic leukaemia. It was found that if such plasma was inoculated intracerebrally into baby mice, a rise in the number of circulating lymphocytes was produced in the peripheral blood. This effect reached its maximum intensity after six days and passed off after fourteen days.

The inoculation of plasma from cases of acute lymphatic leukaemia, multiple myeloma and acute and chronic myeloid leukaemia produced no effect on the lymphocyte levels of inoculated mice.

The effect was believed to have been produced either by a stimulation of lymphocyte production or an induced prolongation of the life span of preformed lymphocytes.

It is the purpose of the present paper to present the results obtained from an examination of a wider range of haematological and other disorders. Lymphocytosis stimulating activity has been detected in the plasma of patients suffering from two other disease states—lymphosarcoma and myelofibrosis.

It has been found that the plasma lymphocytosis stimulating activity (L.S.A.) diminishes following transfusions of whole blood and rises following the intramuscular injection of adrenalin.

Concurrent administration of cortisone or oestrogens to inoculated mice prevents the development of a subsequent lymphocytosis. The lymphocytosis stimulating factor is heat-labile but withstands freeze-drying.

MATERIAL AND METHODS

Blood

Blood was obtained by sterile venepuncture. Ten ml. of blood were withdrawn and placed in sterile heparinised bottles. This blood was centrifuged at 3000 r.p.m. for ten minutes and the supernatant plasma removed. This plasma was either used immediately or stored at −15° C.

Mice

Mice used were those of the Hall Institute stock. This colony has been inbred for many years, but is not genetically homozygous.

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The mice were inoculated when between 24 and 36 hours old. Care needs to be taken in determining the age of the mice as the blood picture of baby mice alters rapidly in the first week of life (Metcalf, 1956).

Following inoculation, the mice were returned to their mothers and left untouched until blood counts were performed on them.

The number of mice in each litter was limited to a standard size of six per nursing mouse. Provision of cotton wool nests for the mice was found useful in minimising deaths from neglect immediately following inoculation.

Inoculation

The mice were inoculated intracerebrally with 0·01–0·02 ml. of plasma using gauge 26 needles and graduated 0·25 ml. syringes.

Inoculations were made in a parasaggital plane midway between the ear and the eye.

The mortality from inoculation was low. Excessive amounts of heparin in the plasma were found to be rapidly lethal to mice of this age and an upper limit of 100 units per 10 ml. of blood was found to be suitable.

Blood counts

Blood counts were performed at a fixed time in the day in all cases.

Blood was obtained from the mice on the sixth post-inoculation day by cutting off the distal centimetre of the tail with a pair of sharp scissors. A free flow of blood resulted from which blood films were made. These films were coded and stained with Leishmann's stain.

Differential white cell counts were performed in the standard fashion and the lymphocyte : polymorph ratio calculated.

In general twelve to eighteen mice were used for the estimation of L.S.A. in each plasma sample. The mean of these estimations was taken as the L.S.A. for that plasma sample.

Where absolute white cell counts were performed, tail blood was again used as above, and counts were made in the standard manner using a modified Levy haemocytometer.

Statistical Analysis

Analysis for statistically significant differences in the L.S.A. values of various plasma samples was performed using the Student " t " series method for small groups.

RESULTS

Preliminary considerations

It was previously noted (Metcalf, 1956) that the absolute lymphocyte count of inoculated mice rose progressively following the inoculation of plasma showing lymphocytosis stimulating activity (L.S.A.).

This effect was at its most constant, and reached its maximum, on the sixth post-inoculation day. Serial daily blood counts are laborious and time-consuming and do not permit the screening of large series of plasma samples or detailed investigations on a particular plasma sample.

It was, therefore, decided to restrict the measurement of L.S.A. to estimations performed on the sixth post-inoculation day.

The lymphocyte : polymorph ratio as determined from differential white counts on stained blood films proved a more reliable and consistent means of estimating L.S.A., than the absolute lymphocyte count. This method of estimation was adopted as the index of lymphocytosis stimulating activity (L.S.A.) throughout the investigation.

Lymphocytosis stimulating activity in various diseases

It was established from a large series of normal plasma samples that the L.S.A. of normal plasma varied between 1·0 and 3·1 with a mean figure of 2·5. This range, in fact, represented the range of the lymphocyte : polymorph ratios of normal uninoculated mice of this age.

It would seem, therefore, that the intracerebral inoculation of normal plasma has no effect on the white cell levels in the peripheral blood—on the sixth post-inoculation day at least.

In Table I are presented the results obtained from the screening for L.S.A. of plasma samples from various types of leukaemia. In Table II are listed, in abbreviated form, the results obtained from a survey of other disease states involving the haemopoietic system.

It may be seen that, of the various types of plasma examined, only three disease groups showed any lymphocytosis stimulating activity. These were : chronic lymphatic leukaemia, lymphosarcoma and myelofibrosis.

All of the eighteen cases of chronic lymphatic leukaemia examined showed lymphocytosis stimulating activity with the exception of one case (No. 2) in

TABLE I.—*Lymphocyte/Polymorph Ratios on the Sixth Day. Results using Plasma from Various Types of Leukaemia*

Patient.	Normal mice.	Normal human Plasma.	Chronic lymphatic leukaemia.	Lympho-sarcoma.	Myelo-fibrosis.	Chronic myeloid leukaemia.	Acute myeloid leukaemia.	Acute lymphatic leukaemia.	Multiple myeloma.
1			4·2	3·9	4·7	2·3	2·6	3·1	2·3
2			*2·3	4·0	2·4	1·7	2·3	3·2	2·3
3			5·2	3·5	3·7	3·0	2·0	2·8	2·3
4			4·5	3·8	2·7	2·3	3·1	2·5	2·8
5			5·5	4·7	4·0	2·0	2·8	3·1	—
6			5·1	3·3	4·3	3·1	2·5	1·7	—
7			4·6	4·1	3·1	2·5	2·6	2·7	—
8			3·2	3·4	2·8	2·8	2·3	2·4	—
9			4·6	3·7	—	—	2·5	2·6	—
10			3·9	4·4	—	—	2·3	2·7	—
11	100 Estimations Range 1·0–3·1	100 Estimations Range 1·0–3·1	4·3	3·7	—	—	2·8	—	—
12			4·4	3·2	—	—	2·9	—	—
13			4·6	3·9	—	—	2·7	—	—
14			3·5	4·0	—	—	2·8	—	—
15			4·9	3·3	—	—	2·6	—	—
16			3·4	5·4	—	—	—	—	—
17			*3·0	—	—	—	—	—	—
18			4·1	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—
Average L.P ratios	2·5	2·6	4·2	3·9	3·5	2·5	2·6	2·7	2·4

* See text.

TABLE II.—*Summary of Results Obtained After Screening Various Diseases showing Haematological Manifestations*

Disease.	No. of cases.	No. of cases showing plasma L.S.A.
Glandular fever	16	0
Whooping cough (> 30,000 lymphocytes/cu. mm.)	4	0
Reticulum cell sarcoma	3	0
Hodgkin's disease	4	0
Aplastic anaemia	4	0
Pernicious anaemia	2	0
Tuberculosis	2	0

clinical remission and one case (No. 17) complicated by a secondary pyaemia during the course of which, the patient's lymphocyte count fell from 55,000 to 5,000.

As described previously, (Metcalf, 1956) the actual level of L.S.A. observed roughly paralleled the clinical activity of the disease at the time of examination.

Of sixteen cases of lymphosarcoma examined, all showed moderate levels of L.S.A. Included in this group are two cases of follicular lymphoma. Three cases of reticulum-cell sarcoma examined showed no L.S.A.

Of considearble interest was the unexpected finding of L.S.A. in the plasma of five of eight cases of myelofibrosis examined. There have previously been no clinical observations made relating myelofibrosis with the lymphatic leukaemia-lymphosarcoma group of diseases, although the association of the disease with myeloid leukaemia is well recognised. The possible relationship between this plasma lymphocytosis stimulating factor and the hypothetical stem-cell stimulating factor in myelofibrosis will be discussed later.

It was realised that the groups of disease states showing plasma L.S.A. were composed of patients who were middle aged and elderly, and as such, this age range was not covered by the normal control group and the other disease groups.

Plasma samples from patients of this age group were, therefore, tested. Included in this group were a number of cases of carcinoma of various sites.

The results obtained are shown in Table III. It may be seen that in no instance was detectable L.S.A. found in the plasma samples.

In summary, it was found that lymphocytosis stimulating activity could be detected in the plasma of sixteen of eighteen cases of chronic lymphatic leukaemia, sixteen of sixteen cases of lymphosarcoma and five of eight cases of myelofibrosis. In no other disease state examined was there detectable lymphocytosis stimulating activity in the plasma.

Effect of Transfusion on Plasma L.S.A.

It has been estimated by Tivey (1954) that clinical remissions in acute leukaemia occur in 20 per cent of cases following large or exchange transfusions. Patients suffering from chronic lymphatic leukaemia are also often benefited by whole blood transfusions.

Whitby (1951) has claimed that remissions may also be induced by transfusion of fresh plasma and has postulated the presence of a factor in normal plasma which, on administration, corrects a deficient or imbalanced state existing in these patients.

TABLE III.—*Lymphocyte/Polymorph Ratios of Mice Inoculated with Plasma from Middle-Aged and Elderly Patients with Various Diseases*

Patient.	Age.	Disease.	No. of estimations.	L/P ratios of inoculated mice (normal range 1·0–3·1).
CA	60	Cancer of lung	6	2·7
DO	70	Cancer of oesophagus	6	2·3
SWA	67	Cancer of stomach	6	2·6
RIG	62	Cancer of lung	6	2·5
HOR	72	Cancer of lung	6	2·7
RO	56	Cancer of lung	6	2·2
FRA	61	Cancer of stomach	6	2·6
DAL	64	Cancer of pancreas	6	2·7
LIS	68	Coronary occlusion	6	2·6
TAY	62	Chronic pancreatitis	6	2·6
FOR	53	Cirrhosis of liver	6	2·3
COL	70	Diabetes	6	2·4
LIN	58	Rh. heart disease	6	2·8
WAL	57	Rh. heart disease	6	2·7
VIR	69	Coronary occlusion	6	2·4
JO	75	Haematemesis	6	2·6

It was of interest therefore, to determine the plasma L.S.A. of selected cases before and after transfusions of whole blood.

For this purpose only previously untreated cases or cases in which other therapeutic procedures had been suspended for some time were used in the estimations. The group examined consisted of four cases of chronic lymphatic leukaemia and two of lymphosarcoma.

The results obtained are shown in Fig. 1. Of the six cases examined, a definite and immediate fall in plasma L.S.A. levels occurred following transfusion in four of the cases. In remaining two cases there were also slight falls which did not reach the level of significance. The volume of blood administered in each case is indicated by the figures on the graphs. With the exception of the patient who received one pint of blood by a direct transfusion, the source of blood was a standard blood bank pool.

The depression in L.S.A. levels occurred promptly following transfusion and appeared to persist for at least one to two weeks.

In five of the six cases, the clinical condition of the patients showed no improvement other than that expected following the restoration of an adequate haemoglobin level. The remaining case showed a return towards normal of W.B.C. count, platelets and lymph node size. This improvement lasted for two months, thereafter the patient's clinical condition deteriorated.

In an attempt to clarify the mechanism by which this depression of L.S.A. levels was induced, the effect of *in vitro* addition of normal plasma to the pre-transfusion active leukaemia plasma, was investigated. Equal volumes of normal and leukaemic plasma were mixed at room temperature and allowed to stand for one hour. The mixture was then inoculated into test mice. In addition, serial dilutions in saline of the same active leukaemic plasma were made and tested for detectable L.S.A.

The results are shown in Table IV.

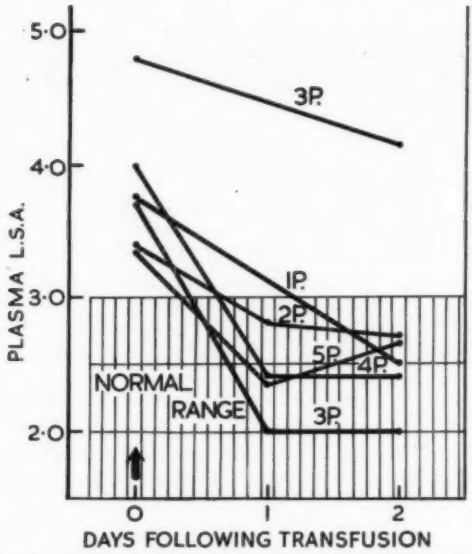

FIG. 1.—Plasma L.S.A. levels following transfusion of four cases of chronic lymphatic leukaemia and two cases of lymphosarcoma. Volume of blood given indicated in pints.

TABLE IV.—*Effect of dilution on plasma L.S.A.*

Type of Plasma.	Plasma L.S.A.
Pre-transfusion plasma	4·0
Plasma following transfusion of four pints of blood (dilution of patient's plasma = 1 : 1·5)	2·4*
Pre-transfusion plasma + normal plasma (dilution of patient's plasma = 1 : 2)	3·8
Saline dilutions of pre-transfusion plasma, 1 : 2	3·5
1 : 5	2·7*
1 : 10	2·8*

* Significantly different from activity of pre-transfusion plasma.

It may be seen from the table that the transfusion of four pints of whole blood to the patient, producing at most a dilution of the patient's plasma of 1 : 1·5, was followed by a sharp fall in detectable L.S.A.

The *in vitro* dilution of the pre-transfusion plasma with normal plasma to a dilution of 1 : 2 produced no such effect.

Similarly, saline dilutions of the pre-transfusion plasma showed no diminution in L.S.A. until a dilution of 1 : 5 was reached.

It would seem unlikely, therefore, that the post-transfusion depression of plasma L.S.A. is due simply to dilution of the patient's blood. The effect may be dependent on secondary events occurring in the patient following transfusion.

Effect of Adrenalin on Plasma L.S.A.

The elevation of blood lymphocyte levels following injections of adrenalin is well known (Michael, 1949; White, Ling and Klein, 1950; Samuels, 1951).

In the hope of demonstrating a similar effect on plasma L.S.A., selected patients suffering from chronic lymphatic leukaemia were given intramuscular injections of adrenalin and the plasma L.S.A. levels estimated.

Four patients were selected with moderately low plasma L.S.A. levels. They were given intramuscular injection of 12 m. of 1/1000 adrenalin. Sterile heparinised blood was collected at 5-minute intervals following the injection. These samples were then tested for plasma L.S.A. in the usual manner.

Two of the patients developed haemolytic anaemia as a complication of their leukaemic state. These patients were subjected to splenectomy. The adrenalin tests were repeated following splenectomy and again after the patients had been placed on routine doses of cortisone (maintenance dose 75 mg. per day).

The results obtained are presented in Fig. 2.

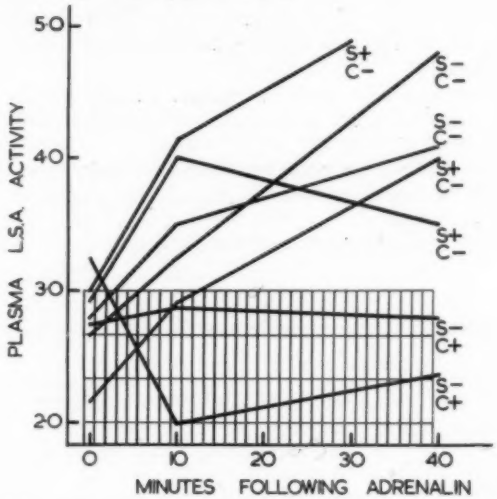

FIG. 2.—Plasma L.S.A. levels following intramuscular adrenalin: S +, with spleen intact. S −, following splenectomy. C +, on cortisone therapy. C −, not on cortisone therapy.

It was found that a definite rise in plasma L S.A. followed the intramuscular injection of adrenalin. This rise did not run parallel with the transitory lymphocytosis exhibited by these patients. The lymphocytosis reached its maximum and returned to normal levels within the first ten minutes. On the other hand, as may be seen in Fig. 2, the plasma L.S.A. rose steadily throughout the observation period of 40 minutes.

The exact duration of the elevation of plasma L.S.A. was not determined, but plasma L.S.A. levels had returned to the pre-injection levels when the patients were retested a week after the test.

Splenectomy, in the two patients examined, appeared to have no influence on the adrenalin-induced rise in plasma L.S.A.

However, when these patients were placed on cortisone therapy, and the experiment repeated, no elevation of plasma L.S.A. occurred following the adrenalin injections.

It would appear, therefore, that adrenalin is capable of stimulating either the production or release of L.S.A. This effect can occur in the absence of the spleen and presumably, therefore, the spleen, if involved at all in the production of L.S.A., is not the sole source of this factor.

Further, it seems that cortisone, in the doses used, is capable of inhibiting this production or release of excessive amounts of L.S.A. by adrenalin.

Effect of Cortisone and Oestrogen on the Lymphocytosis Response in the Mouse

In view of the known depressive effect of cortisone on blood lymphocyte levels (Dougherty and White, 1947), the effect of the simultaneous administration of active plasma and cortisone on the test mice, was investigated.

To check the effect of cortisone alone, day-old mice were injected subcutaneously with 0·1 mg. of cortisone acetate daily for three days. When examined at seven days of age, a significant fall in the blood lymphocytes was found to have occurred.

Plasma of known activity was inoculated intracerebrally, simultaneously with the first cortisone injections, and lymphocyte counts performed on the mice six days later.

The mice did not show the customary lymphocytosis, the lymphocyte counts were within normal limits, but were slightly higher than the counts on mice treated with cortisone alone.

Table V shows the details of one such experiment.

TABLE V.—*Effect of Cortisone on Plasma-Induced Lymphocytosis in Mice*

Estimation.	Number of mice per group.	Untreated mice.	Mice + plasma.	Mice + cortisone.	Mice + cortisone + plasma.
Average L/P ratio, normal range 1·0–3·1	18	2·5	4·4	1·2	1·7
Average total polymorphs/cu. mm.	18	1000	800	1300	1500
Average total lymphocytes/cu. mm.	18	2500	3800	1600	2500

The significance of these results is not clear. They may represent the result of a mutual antagonism between cortisone and the lymphocytosis stimulating factor. A more likely interpretation is that the cortisone has, by a direct destructive effect, depressed the level of circulating lymphocytes in both the untreated mice and the mice showing a plasma-induced lymphocytosis.

The effect of oestrogen on the lymphocyte levels of normal and plasma-treated mice was next investigated.

Day-old mice were inoculated subcutaneously with 50 i.u. of oestroform (oestradiol benzoate). No detectable effect on lymphocyte levels was observed on the sixth post-injection day

When active plasma was inoculated simultaneously with the subcutaneous oestroform, the expected lymphocytosis did not develop.

Table VI shows the results obtained.

TABLE VI.—*Effect of Oestrogen on Plasma-Induced Lymphocytosis*

Experiment.	Number of mice per group.	Normal mice.	Mice + plasma.	Mice + oestrogen.	Mice + plasma + oestrogen.
I	10	2·7	3·8	2·9	2·4
II	20	2·6	3·8	2·8	3·0
III	20	2·6	4·1	2·8	2·2

It may be seen that whilst oestrogen alone had no significant effect on the normal L/P ratio, it prevented the elevation of the L/P ratio following the inoculation of active plasma.

Following on these results, a large group of mice of both sexes was inoculated with a standard volume of active plasma. No observable difference in the level of the lymphocytic response was found between the two sexes. This was not surprising in view of the known sexual immaturity of mice of this age (cf. basis of the Aschheim-Zondek test on considerably older mice).

Effect of heat and freeze-drying on plasma L.S.A.

It was found that the lymphocytosis stimulating activity of active plasma samples was lost following heating of the plasma to 60° C. for 15 minutes.

It was also found that the L.S.A. level of an active plasma sample withstood storage at −15° C. for several months at least.

The activity of plasma samples was also found to withstand freeze-drying.

These results are listed in Table VII.

TABLE VII.—*Effect of heat and freeze-drying on plasma L.S.A.*

Type of plasma.	L.S.A.
Plasma untreated	3·8
Plasma heated to 60° C. for 15 minutes	2·5
	2·5
	2·7
Plasma stored at − 15° C. for 3 months	3·8
	3·6
	3·8
Plasma after freeze-drying	3·7
	3·9

DISCUSSION

The present work has confirmed the original finding that a high percentage of patients, suffering from uncomplicated chronic lymphatic leukaemia, has present in their plasma a factor capable of stimulating a lymphocytosis in young mice.

In addition, two other disease states, lymphosarcoma and myelofibrosis, appear to show similar plasma activity.

The finding of plasma L.S.A. in lymphosarcoma patients is in accord with clinical and experimental experience that the disease is closely allied to, if not identical with chronic lymphatic leukaemia.

There is no such obvious relationship between the two foregoing diseases and myelofibrosis. It has been postulated by Dameshek (1951) and others that myelofibrosis represents a disease in which the stem cells have been subjected to continued and excessive stimulation. The effects of such stimulation may be channelled either towards excessive activity of fibroblasts and osteoblasts or

towards the eventual development of myeloid leukaemia. This stimulation may be mediated by a circulating factor present in the plasma. Such plasma might induce a lymphocytosis in an experimental animal whose only possible response to stimulation of its stem cells was a lymphocytic one. Such a situation may exist in baby mice, where it is known that, at this stage in their development, the blood picture is undergoing conversion to a lymphocytic type.

At the present time, however, the detection of plasma L.S.A. in myelofibrosis must remain an empirical observation.

The interpretation of the true biological function of the plasma lymphocytosis stimulating factor in these disease states remains difficult.

The assumption, that the function of the factor in the human patient is identical with the observed effect in mice, is to some extent supported by the findings of other workers using similar material in members of two other species—guinea-pigs and human volunteers.

Foster and Miller (1950) reported a stimulation of activity in the lymph nodes, and a variable lymphocytosis in guinea-pigs following intraperitoneal inoculation of plasma from cases of lymphatic leukaemia.

Similarly, Oliva and Tramontana (1950) reported transient elevations of circulating white cell levels following the intravenous infusion of human volunteers with leukaemic plasma.

It seems reasonable to assume, therefore, that the lymphocytosis stimulating factor in the plasma of these patients may be involved either in the stimulation of lymphocyte production or in the maintenance of elevated lymphocyte levels.

It was of interest that the plasma of patients with glandular fever and whooping cough, who were exhibiting a lymphocytosis, showed no detectable L.S.A. This was also found to be the case with plasma from cases of acute lymphatic leukaemia. These patients fall into a young age group and it is possible that the lymphocytosis stimulating factor may not appear in detectable amounts in the plasma at this age.

Alternatively, the amount of circulating factor required to produce a given level of lymphocytosis may be smaller in younger patients, and not detectable by the present technique.

The possibility remains that the factor is a specific one and only produced in certain disease states.

The effect of transfusions in lowering plasma L.S.A. is of interest. It may represent a specific *in vivo* neutralisation of the lymphocytosis stimulating factor by factors present in normal blood, but it is perhaps more likely to result from a secondary *in vivo* reaction produced by the transfusion. Such a reaction may be the release of excessive amounts of cortisone. In accord with this hypothesis is the apparent antagonism by cortisone of the elevation of plasma L.S.A. following the intramuscular injection of adrenalin.

At this stage, neither the antagonistic effect of cortisone nor the stimulating effect of adrenalin, on plasma L.S.A. levels, can be more than noted. Whether the responses to these artificial stimuli represent normally occurring control mechanisms of plasma L.S.A. levels in the body, is purely speculative.

Of similar significance is the finding of an apparent antagonism by oestrogens of the L.S.A. effect. It is known that there is a sharp sex difference in the incidence of the disease, males predominating. What relation there is between these observations, can again only be speculated upon.

In summary, the relation, between plasma L.S.A. and the disease states in which it has been found to occur, may be :

(*a*) An unrelated phenomenon, at most representing a semi-specific by-product of the disease state.

(*b*) A compensatory state by which normal lymphocytic activity is maintained following involvement of the body's normal complement of lymphocytic tissue by disease.

(*c*) An aetiological relationship in which the continued presence of the lymphocytosis stimulating factor leads to over-activity of the patients lymphopoietic tissue with the final development of the leukaemic or myelofibrotic state.

The potential use of the L.S.A. test as a diagnostic procedure is worthy of consideration, but the value of such a test can only be assessed after a more extensive screening of various disease states has been performed.

SUMMARY

1. A factor has been detected in the plasma of several disease states which is capable of inducing a lymphocytosis in baby mice. These disease states are :— chronic lymphatic leukaemia, lymphosarcoma and myelofibrosis.

2. The amount of the factor detectable in the patients' plasma is depressed by transfusion of whole blood and during clinical remissions. It is elevated by the intramuscular injection of adrenalin.

3. Both cortisone and oestrogens appear to suppress the lymphocytosis effect of active plasma samples in mice.

4. Plasma activity is heat-labile but withstands freeze drying.

5. The significance of these findings is discussed.

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I am indebted to Mrs. J. Ramsay for the graphs.

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THE THYMIC ORIGIN OF THE PLASMA LYMPHOCYTOSIS STIMULATING FACTOR

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It was shown (Metcalf, 1956*a*, 1956*b*) that the plasma from several disease states was capable of inducing a lymphocytosis when inoculated into baby mice.

The disease states, in which this plasma activity was detected, were: chronic lymphatic leukaemia, lymphosarcoma and myelofibrosis.

Certain factors influencing this plasma lymphocytosis stimulating factor were also noted. Plasma activity was depressed during natural remissions and following transfusions of whole blood. Plasma activity rose following intramuscular injections of adrenalin. The lymphocytosis effect in inoculated mice was inhibited by pre-heating of the plasma to 60° C. or by the concurrent administration of cortisone or oestrogen.

To elucidate the relationship between the plasma factor and the disease states in which it is found, an attempt was made to determine the organ or organs producing or storing this factor.

It has been found that emulsions of normal human thymus tissue possess a lymphocytosis stimulating activity when injected into baby mice. Mouse thymus emulsions show similar activity.

This thymic substance is closely related to, or identical with the lymphocytosis stimulating factor found in the plasma of the above-mentioned disease states.

Preliminary investigations have shown that the thymus in these disease states has a higher than normal content of lymphocytosis stimulating material.

MATERIAL AND METHODS

Human material

Specimens from normal human organs were obtained during autopsies on persons dying from accidental causes. Similar specimens were also obtained from patients dying from chronic lymphatic leukaemia and lymphosarcoma.

In all cases the material was obtained within twelve hours of death.

All specimens were removed by a no-touch technique using sterile instruments.

Organ emulsions were prepared from these specimens by grinding 0·25 g. of the tissue with sterile silica in pestle and mortar. The ground tissue was then emulsified to a final dilution of 1 : 20 using sterile normal saline with added penicillin and streptomycin (25 units of each per ml.).

The emulsions were centrifuged at 3000 r.p.m. for five minutes to remove the silica and large cellular debris. The supernatant fluid was either used immediately or stored at −20° C.

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Mouse material

Mice used were those of the Hall Institute stock. Standard 1 : 20 organ emulsions were prepared as described above from both normal mice and mice with naturally occurring chronic lymphatic leukaemia.

Test mice

The mice used for testing the activity of organ emulsions were again those of the Hall Institute stock. These mice have been interbred for a number of years, but are by no means genetically homozygous.

Method of testing

Organ emulsions from the above sources were tested for lymphocytosis stimulating activity in the following manner.

Three litters of 1–2 day old mice (18 mice) were used for each organ emulsion tested.

Each mouse was inoculated intracerebrally with 0·03 ml. of organ emulsion using 0·25 ml. graduated syringes and Gauge 25 or 26 needles. Injections were made midway between the eye and the ear.

The mice were then returned to their mothers. Six days after inoculation, white cell counts were performed on all mice using blood obtained from the tail, by cutting off the tip of the tail with a pair of sharp scissors.

The flow of blood produced by this method can be regulated by the firmness with which the hind quarters of the mouse are held.

Rapidly-spread blood films were prepared, stained with Leishmann's stain and differential white cell counts made according to a standard pattern. The lymphocyte/polymorph ratio was calculated and the mean L/P ratio of the group of 18 mice determined.

Absolute white counts where performed, were done in standard fashion using a modified Levy haemocytometer.

The heart blood of the mouse contains many fewer white cells per unit volume than the peripheral blood (Fekete, 1941). This fact is of importance when dealing with baby mice where the total blood volume is small.

Absolute white cell counts and blood films should, therefore, be made from the first drop of blood obtained from the tail. In addition, the white cell diluting pipette should be filled first to minimise the variability of results when the heart blood dilutes the peripheral blood.

Absolute white cell counts cannot be repeated on any one mouse because of these considerations. Further, the mice appear to develop a brisk polymorphonuclear leucocytosis in response to the trauma of tail-cutting. This renders meaningless any subsequently performed differential white cell count.

Despite these apparent limitations the method of estimation is simple and capable of giving reproducible results.

Calculation of organ content of L.S.S.

Where a standard 1 : 20 organ emulsion was found to have a lymphocytosis stimulating activity, an estimation of the organ content of this lymphocytosis stimulating substance (L.S.S.) was carried out in the following manner.

Serial dilutions of the organ emulsion were tested and the highest dilution, at which a lymphocytosis could be produced, was determined.

The organ content of L.S.S. was then calculated in mouse units, where 1 mouse unit (M.U.) = 0·03 ml. of highest dilution of the organ emulsion producing a lymphocytosis. For example, if 0·25 g. of an organ weighing 1 g. was found to induce a lymphocytosis at a dilution of 1/250, then

$$\text{Total organ content of L.S.S.} = \frac{1}{0{\cdot}25} \times 250 \times 30\,\text{M.U.} = 30{,}000 \text{ mouse units.}$$

Tissue cultures

Maitland type tissue cultures of mouse thymus were prepared in the following manner.

Mice were killed by beheading and the thymuses were removed aseptically and washed in Earle's solution.

The thymuses were coarsely minced with sharp scissors and transferred to 5 × $\frac{5}{8}$ inch roller tissue culture tubes.

The number of thymuses per tube was either three 8-day thymuses or ten 2-day thymuses.

Two ml. of nutrient fluid were then added to each tube. The composition of this fluid was;

Earle's Solution
Penicillin } 50 Units of each per ml.
Streptomycin }

The tubes were rotated at 60 r.p.hour at 36° C.

RESULTS

Lymphocytosis stimulating activity of various human and mouse tissues

As a preliminary screening procedure, saline emulsions of various human and mouse organs were prepared at a standard 1 : 20 dilution. These emulsions were then injected into groups of eighteen 2-day old mice and the mice examined six days later.

The presence or absence of a lymphocytosis for mice of this age was determined by performing absolute and differential white cell counts on tail blood. The upper limits of the normal white cell count for mice of this age have been established as a total lymphocyte count of 2500 per cu.mm. and a lymphocyte/polymorph ratio of 3·0 (Metcalf, 1956*a*).

As cases of chronic lymphatic leukaemia in man and mice became available, organs from these sources were also screened in the above fashion.

The results obtained are presented in Table I.

It may be seen that, of the normal human and mouse organs tested, only two, the thymus and the thyroid, showed any lymphocytosis stimulating activity.

This held also for those cases of chronic lymphatic leukaemia which were examined. However, in these cases, the plasma or serum also exhibited a lymphocytosis stimulating activity.

The lymphocytosis following injections of thyroid emulsions was only slight in degree. For reasons to be discussed later, it appears likely that the effect occurred as a secondary phenomenon due to stimulation by the thyroid emulsion of the inoculated mice's own thymus glands.

TABLE I.

Type of tissue.	Tissues showing lymphocytosis stimulating activity.	Tissues showing no lymphocytosis stimulating activity.
Normal human	Thymus Thyroid	Plasma or serum Lymph Node Liver Spleen Heart Lung Adrenal Bone marrow Brain Pituitary Kidney
Normal mouse	Thymus Thyroid	Serum Lymph node Liver Spleen Heart Lung Kidney Brain
Chronic Lymphatic Leukaemic Human	Thymus Plasma or serum Thyroid	Lymphocytes (circulating) Lymph node Leukaemic masses Liver Spleen Heart Lung Adrenal Bone marrow Brain Pituitary Kidney
Chronic Lymphatic Leukaemic Mouse	Thymus Serum Thyroid	Lymph node Leukaemic masses Liver Spleen Heart Lung Brain Kidney

Emulsions of organs other than the thymus and the thyroid did not alter the peripheral blood picture from normal with the exception of adrenal gland emulsions which produced a lymphopenia.

A brief survey of normal human thymuses from persons of various ages was carried out. This revealed that thymus tissue at all ages possesses a lymphocytosis stimulating activity for mice. However, the total thymic content of the lymphocytosis stimulating substance (L.S.S.) fell considerably with advancing age.

The thymic L.S.S. content of a small series of children varying in age from several days to one year, was estimated at approximately 200,000 mouse units (1 mouse unit = 0·03 ml. of the highest dilution of thymus emulsion showing lymphocytosis stimulating activity).

The thymus was not always demonstrable in elderly people. Where definite thymic tissue could be demonstrated by naked eye dissection, the L.S.S. content was found to be in the region of 1500 M.U.

This survey of human thymuses was not pursued exhaustively due to the large number of mice needed for estimating the activity of each dilution of thymus emulsion. However, it was established beyond doubt that the normal human thymus contains a substance (L.S.S.) capable of producing a lymphocytosis in baby mice, and that the thymic content of L.S.S. decreased with advancing age.

Similar titrations were performed on normal mouse thymuses. Here the situation was somewhat different. The thymic content of L.S.S. in the newborn mouse was very low (< 2 M.U.). During the first week of post natal life, the thymic content rose sharply reaching the maximum in young adult life (> 3 months). Thereafter the thymic content decreased to levels of the order of 100 M.U.

The increasing thymic content of L.S.S. during the first week of life was of interest, as it is during this period that the peripheral blood picture of the mouse changes from a polymorphonuclear one to one showing a preponderance of lymphocytes.

A preliminary investigation of the thymus in chronic lymphatic leukaemia in both man and mice has shown that there is a considerably higher thymic content of L.S.S. than in comparable normal thymuses.

These results have been set out in Fig. 1 and 2.

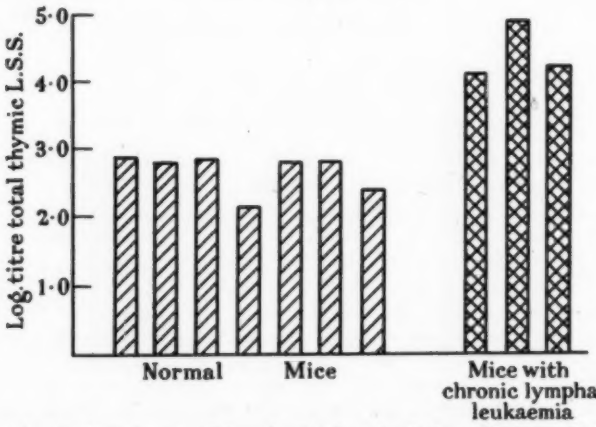

FIG. 1.—Total thymic contents of lymphocytosis stimulating substance (L.S.S.) expressed logarithmically in mouse units. Each column represents a single mouse.

Fig. 1 shows the total thymic L.S.S. content of a number of leukaemic mice and a comparable group of normal mice of the same age. Thymic titres are expressed in mouse units on a logarithmic scale, each column representing a single mouse.

Fig. 2 shows the results obtained from a similar survey of human cases of chronic lymphatic leukaemia. In this instance, due to considerable variation in the ages of the patients, the thymic L.S.S. titres have been compared individually

with normal thymus L.S.S. titres of persons of the same age and sex. The first and third cases were aged 60 years and the second 35 years. It may be seen that, for the younger pair, the total thymic L.S.S. content of both the normal and leukaemic person was high.

In the human cases, the effect of therapeutic procedures, particularly irradiation, on the histology and function of tissues in the thymic area, made an assessment of previous thymus function difficult.

In some cases, dense fibrotic tissue in the thymic region made it impossible to identify and dissect free the thymus.

For this reason, the survey was limited to cases which had had no previous therapy directed to the mediastinal area.

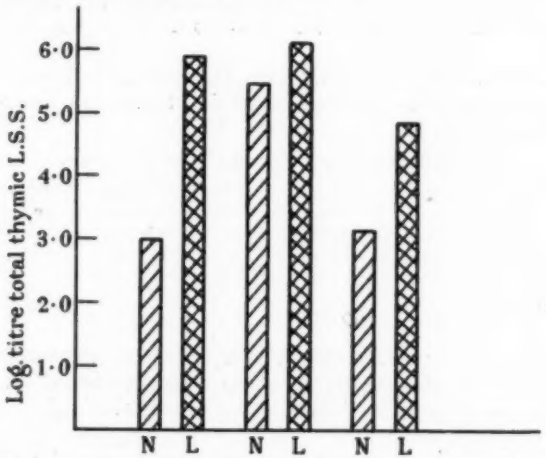

FIG. 2.—Total thymic contents of lymphocytosis stimulating substance (L.S.S.) expressed logarithmically in mouse units. Each column represents a single case. L = Chronic lymphatic leukaemia. N = Normal humans of same age and sex.

In both the uncomplicated human cases and the leukaemic mice, the thymus was larger than normal, sometimes markedly so. However, the variable extent to which the gland was infiltrated with lymphocytic tissue, made it difficult to assess the mass of the thymic tissue proper.

No answer could, therefore, be obtained on the question of whether the higher than normal total thymic L.S.S. content was due to a greater thymic cell mass or a higher concentration of L.S.S. per cell.

The increased thymic content of L.S.S. in chronic lymphatic leukaemia is of obvious interest in regard to the relationship between thymic L.S.S. and the lymphocytosis stimulating factor detectable in the plasma of these patients.

Before definite conclusions can be drawn however, the observations on elevated thymic L.S.S. titres need confirmation and amplification with a larger series.

Production of L.S.S. by thymic fragments in tissue culture

Tissue culture experiments using normal mouse thymus were performed in an attempt to determine whether the thymus tissue was actually producing this

L.S.S. or merely acting as a storage organ for material produced elsewhere in the animal.

Thymuses were removed aseptically from normal 7 day-old and 2 day-old mice and coarsely minced with sharp scissors. The thymic fragments were rinsed in Earle's solution and set up in roller type Maitland tissue cultures as described above.

After 24 hours incubation in the case of the 7 day-old thymuses and 5 days incubation for the 2 day-old thymuses, the tubes were removed from the incubators.

The tissue culture fluids were removed and the thymus fragments were emulsified in normal saline. Both sets of material were then tested for L.S.S. and, if detectable, the titre was estimated by serial dilution of the original material.

Control 7-day and 2-day thymuses were titrated for L.S.S. content in the standard fashion.

The results obtained are listed in Table II. In the post-incubation column are shown the combined total titre of tissue fragments and tissue culture fluid.

TABLE II.—*Mouse Thymus Tissue Culture*

Type of thymus.	Days of incubation.	Pre-incubation titre in mouse units.	Post-incubation titre in mouse units.
7-day thymus	1	500	3000
2-day thymus	5	<6	>120
2-day thymus	5	<6	>120
2-day thymus	5	<6	>120

It may be seen that a definite rise in total thymic content of L.S.S. occurred following tissue culture particularly in those cultures carried on for five days. This presumably was the result of an actual production of L.S.S. by the thymus tissue fragments.

Secretion of L.S.S. from the thymus fragments into the overlying tissue culture fluid appeared to have taken place, as in each instance, approximately 50 per cent of the combined post incubation L.S.S. was present in the tissue culture fluid. However, this could equally well have been the result of the considerable shedding of intact and damaged thymic cells into the overlying fluid which occurs during this type of tissue culture.

Type of thymic cell producing L.S.S.

An attempt was made to identify the thymic cell type producing the L.S.S. detectable in human and mouse thymuses.

The thymus contains two main cell types—small round cells with the morphological appearance of lymphocytes and larger epithelial type cells.

Thymic tissue also contains a network of reticulum cells but this tissue was considered unlikely as the source of the L.S.S. in view of the absence of detectable L.S.S. in lymph nodes and splenic tissue.

The mouse thymus is suitable for an approach to this problem because of the arrangement of thymic tissue into a sharply defined cortex of small round cells and a medulla of the large epithelial cells.

Mouse thymuses were removed and frozen solid in Petri dishes placed on blocks of dry ice. In this rigid state it was possible to shave off cortical tissue with a

sharp scalpel. Histological check of these cortical shavings showed no contaminating medulla cells.

It was not possible by this method to obtain medullary tissue free from cortical cells.

Pooled fragments of cortical tissue and medullary tissue with cortical contamination were emulsified and assayed for L.S.S.

Table III shows that whilst the medulla fragments were quite active, the pure cortical emulsions showed no activity.

The implication is strong that the epithelial cells of the thymus are the source of the L.S.S. in this organ.

However, the possibility that cortical cells may play an associated role in the production of L.S.S., although unlikely, has not been excluded.

TABLE III.

Mouse thymus.	No. of experiment.	Lymphocytosis stimulating activity. L/P ratios of inoculated mice (normal range = 2·0–3·1).
Cortex	I	2·5
	II	2·8
Medulla + cortical fragments	I	4·0
	II	4·1

Relationship between plasma L.S.F. and thymus L.S.S.

The thymus in eight day mice normally contains detectable amounts of L.S.S.

No detectable thymus L.S.S. could be found, however, in eight day mice which had been injected at the age of two days with a lymphocytosis stimulating inoculum of mouse thymus emulsion.

This apparent depression of thymus activity was also found to occur following inoculations of human thymus emulsions. More importantly, the same effect was found to have occurred following inoculations of plasma containing the lymphocytosis stimulating factor (L.S.F.).

On the other hand, inoculations of non-active human plasma, emulsions of other organs or thymus emulsions diluted beyond the limit of the L.S.S. titre, produced no alteration in the thymus L.S.S. content of the inoculated mice.

The method used to demonstrate this effect was as follows. Groups of six, 2 day old mice were injected with inocula of various types. Examination of the peripheral blood at the age of eight days was performed to determine the presence or otherwise of a lymphocytosis.

The thymuses were then removed from these mice, emulsified in normal saline to a standard 1 : 20 dilution and tested for lymphocytosis stimulating activity in a further series of baby mice.

The results obtained are recorded in Table IV. The presence of a lymphocytosis or otherwise was determined by calculating the mean value for the lymphocyte/polymorph ratio in the peripheral blood of each group of mice on the sixth post-inoculation day. A ratio greater than 3·1 indicates a lymphocytosis (Metcalf, 1956a).

TABLE IV.

Origin of thymic tissue.	No. of experiment.	No. of mice used for estimation.	L/P ratios following inoculation of thymus emulsions (normal range 2·0–3·1).
Normal 8-day mice	I	20	4·0
	II	18	4·2
	III	12	4·1
Mice inoculated with inactive tissue emulsions	I	12	4·4
	II	14	3·4
	III	18	4·0
Mice inoculated with inactive human plasma	I	12	5·7
	II	14	4·2
Mice inoculated with human thymus emulsions	I	12	2·5
	II	18	2·5
	III	18	2·5
Mice inoculated with mouse thymus emulsions	I	18	2·9
	II	15	2·6
	III	12	2·5
	IV	18	3·1
Mice inoculated with active human chronic lymphatic leukaemic plasma	I	12	2·2
	II	15	2·7
	III	25	2·6
	IV	16	3·0
	V	16	2·7

It may be seen from the table that active leukaemic plasma and thymus extracts were equally effective in depressing the thymic content of L.S.S. of the inoculated mice.

A histological examination of these thymuses was made in an attempt to find a morphological basis for the different activity of the thymuses from inoculated and uninoculated mice.

Thymuses from both groups were fixed in Carnoy's fixative and after sectioning were stained with haematoxylin and eosin, Periodic Acid-Schiff, Masson's stain and Scharlach R (frozen sections).

No constant difference could be detected between the two groups with regard to cell types, cell numbers, mitotic activity or the presence or absence of secretion products.

There was no difference in gross thymic weights between the groups.

It was of interest to determine how long the depression of thymus L.S.S. content lasted, both in the body and when removed to tissue culture conditions.

It was found, by daily testing of thymus emulsions from suitably inoculated mice, that the thymic content of L.S.S. returned to a near-normal level by the twelfth day of age, that is, ten days after the original inoculation.

The effect of tissue culture on the thymic L.S.S. content in inoculated mice was determined in the following manner.

Thymuses were removed from mice previously inoculated with thymus emulsions or active chronic lymphatic leukaemic plasma.

Pools of six thymuses were used in each experiment. The thymuses were divided into halves and one group of halves tested immediately for L.S.S.content.

The other group of halves was cultured in roller tubes for 24 hours as described above. Then, both the tissue culture fluid and an emulsion of the thymus tissue culture were tested for L.S.S.

It was found that, within twenty four hours, the tissue-cultured thymuses from such animals had regained detectable amounts of L.S.S.

These results are shown in Table V.

TABLE V.

Source of thymus.	No. of experiment.	No. of mice per estimation.	L/P ratios of inoculated mice (normal limits 2·0–3·1). Thymus tested immediately.	Tissue cultured thymus after 24 hours.	Tissue culture fluid after 24 hours.
Mice inoculated with active chronic lymphatic leukaemic plasma	I	18	2·6	3·9	3·0
	II	18	3·0	3·6	4·2
	III	15	2·7	3·3	3·9
Mice inoculated with active thymus emulsions	I	16	2·9	3·9	3·8
	II	15	2·5	3·6	—
	III	18	3·1	4·2	—
	IV	18	2·5	3·2	—

It may be seen, therefore, that when the thymuses, showing no detectable L.S.S., were removed from the milieu of the inoculated mice, they rapidly produced detectable quantities of L.S.S.

Experiments were next performed to determine whether or not thymuses with depressed contents of L.S.S. could produce L.S.S. in tissue culture in the presence of active L.S.S. material

The method used was similar in its essentials to the one just described, but in addition tissue cultures of depressed thymuses were set up to which 0·1 ml. of an active 1 : 20 thymus emulsion had been added.

As a control, tubes were also set up containing splenic tissue with and without added thymus emulsion, to guard against the possibility of non-specific adsorption of L.S.S. from the tissue culture fluid.

Control tubes of normal mouse thymus with and without added thymus emulsion were also set up.

After twenty-four hours incubation, the respective tissues were removed from the tubes, washed three times with 5 ml. of sterile saline, and emulsified in normal saline to produce 1 ; 20 emulsions.

These emulsions were then tested in groups of 18 mice for ability to induce a lymphocytosis.

Table VI shows the results obtained. A mean lymphocyte/polymorph ratio greater than 3·1 indicates presence in the inoculated mice of a lymphocytosis.

It may be seen firstly, that the addition of thymus emulsion to tissue cultures of normal thymus did not affect the final L.S.S. content of the tissue. Secondly, there appeared to be no non-specific adsorption of L.S.S. from the tissue culture fluid by the splenic tissue cultures.

However, it will be observed that, whereas the depressed thymuses produced L.S.S. in tissue culture, they did not do so if the tissue culture fluid contained added thymus extract.

TABLE VI.—*Lymphocyte/Polymorph Ratios of Mice Inoculated with Tissues after Tissue Culture for 24 hours.*

Experiment No.	Normal thymus.	Normal thymus + thymus emulsion.	Normal spleen.	Normal spleen + thymus emulsion.	Inoculated thymus.	Inoculated thymus + thymus emulsion.
I	4·0	3·6	2·4	2·6	3·6	2·6
III	4·1	4·2	2·9	3·0	4·2	2·5
III	4·3	4·0	2·7	2·7	3·6	2·7

The above results may be summarised as follows. The inoculation of two day mice with lymphocytosis stimulating material in the form of human plasma or human or mouse thymus causes, in addition to the lymphocytosis, a temporary inhibition of the development of the normal thymic content of L.S.S. in the inoculated mice. These thymuses rapidly develop a normal content of L.S.S. in tissue culture, but fail to do so if thymus emulsion is added to the tissue culture fluid.

These findings have been shown schematically in Fig. 3.

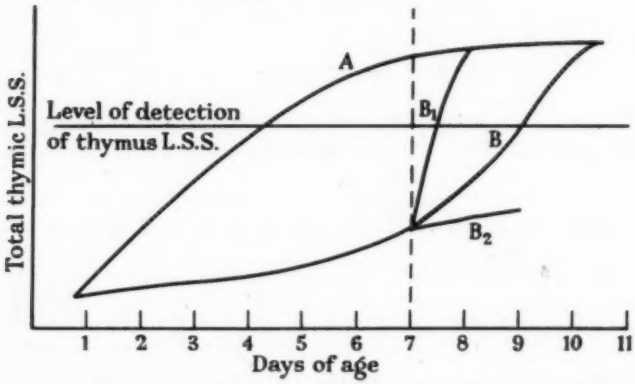

FIG. 3.—Schematic representation of the development of thymic L.S.S. in normal and inoculated mice.

A = Normal development of thymic L.S.S.
B = Development of L.S.S. following inoculation of active L.S.S. material.
B_1 = Tissue culture of B thymuses.
B_2 = Tissue culture of B thymuses with added thymus extract.

One possible interpretation of this phenomenon is as follows. The thymus in 2 day mice is in an early developmental phase during which it contains very little L.S.S.(< 2 M.U.). Normally, perhaps in response to demands from the developing animal, this amount would rise during the first week of life. However, in the inoculated mice, these demands may be met, for a time, by the L.S.S. content of the inoculum. The animals' own thymic tissue, having a lessened functional demand made on it, fails to develop the increase in thymic L.S.S. as rapidly as normal mice.

This situation may be made to persist in tissue culture if the fluid milieu of the cells is made similar to that existing in the inoculated animal, by the addition of the thymus emulsion to the tissue culture fluid.

The phenomenon may thus represent an example of control of the functional development and activity of an organ by the exhibition of extracts of that organ. Similar examples in other organs have been described by Galli-Mainini (1941, 1942) for the thyroid, de Robertis (1940, 1941) for the parathyroid, Kendall (1941, 1942) for the adrenal and by Weiss(1950).

Apart from these considerations, two points were of considerable theoretical interest:

(*a*) the similar depressing effect of thymus emulsions of human and mouse origin—indicating that the active material concerned was functionally similar in both species;

(*b*) the similar depressing effect of plasma from disease states showing plasma lymphocytosis stimulating activity—again suggesting a close functional similarity between the plasma factor and the thymic substance.

This phenomenon has also provided a means of distinguishing between the lymphocytosis stimulating effect of thymus and thyroid emulsions.

As has been described above, the lymphocytosis induced by thymic emulsions is attended by a depression in the thymic content of L.S.S. in the inoculated mice.

When mice are inoculated with thyroid emulsions, a moderate lymphocytosis is produced. However, when the thymus glands of inoculated mice were examined, they were found to have a normal content of L.S.S. These findings are set out in Table VII.

TABLE VII.—*Lymphocyte/Polymorph Ratios in Mice Inoculated with Thymus Emulsions of Various Origins*

Origin of thymic tissue.	No. of mice used for estimation.	Lymphocyte/polymorph ratios following injection of thymus emulsions.
Mice inoculated with thymus emulsions	12	2·5
	18	2·6
	18	2·5
Mice inoculated with thyroid emulsions	16	3·9
	18	3·3
	16	4·0

These findings have been interpreted as indicating that the lymphocytosis occurring following inoculations of thyroid emulsions is a secondary effect resulting from a stimulation of the inoculated mice's own thymuses to greater production of L.S.S.

Effect of heat and oestrogens on thymus L.S.S.

It was noted, Metcalf (1956*b*) that the lymphocytosis following inoculations of active leukaemic plasma samples could be inhibited by preheating the plasma to 60° C. for 15 minutes or by the concurrent administration of oestrogens (50 i.u. oestradiol benzoate per mouse).

When thymic emulsions were subjected to these two procedures, it was found that the lymphocytosis effect was again inhibited.

This is shown in Table VIII.

TABLE VIII.—*Lymphocyte/Polymorph Ratios in Inoculated Mice (normal L/P ratio 2·0–3·1)*

Type of inoculum.	Lymphocyte/polymorph ratio.
Human thymus emulsion	4·0
	4·1
Human thymus emulsion heated to 60° C. for 15 minutes	3·0
	2·7
Human thymus emulsion and oestrogen	2·2

These findings again indicate the close similarity between the plasma factor and thymic L.S.S.

DISCUSSION

Using baby mice as the test animal, emulsions of various normal and leukaemic organs have been tested for lymphocytosis stimulating activity.

Of the organs tested, only two have shown such activity—the thymus and to a lesser degree the thyroid.

The finding that thymus tissue contains a lymphocytosis stimulating substance is in accord with the earlier work of Bomskov and Sladovic (1940). These workers found that oily extracts of normal thymus produced a lymphocytosis when inoculated into rats, guinea pigs and pigeons.

Rehn (1940) has shown that similar preparations can produce a lymphocytosis in man.

Bomskov and Sladovic claimed that the active substance was produced by the epithelial type cells of the thymus, incorporated in lymphocytes and carried throughout the body in this manner.

The present findings have confirmed that the epithelial type cells of the mouse thymus medulla contain such a lymphocytosis stimulating substance (L.S.S.). However, neither thymus cortical tissue, lymphocytes, lymphnodes or spleen have been shown to have any activity.

Furthermore, in those disease states in which the plasma shows lymphocytosis stimulating activity, preparations of lymphocytes, lymphnodes and leukaemic masses are again inactive.

The implication is that the substance found in the thymic epithelial cells is carried throughout the body in the plasma and not the lymphocytes.

Preliminary results have confirmed Bomskov and Sladovic's (1940) claim that young animals have a higher thymic content of L.S.S. than older animals. This has been found to be the case both for mice and humans.

This does not necessarily indicate that the L.S.S. content per cell is higher in the young animal as the thymic cell mass of epithelial cells is very much higher in the young animal.

Maitland-type tissue cultures of mouse thymus have been shown to produce L.S.S. and there appears to be little doubt that the thymus is actually producing this material and not merely storing a substance produced elsewhere in the animal.

The finding that thymic L.S.S. is contained in the epithelial type cells of the mouse thymus throws light on the observations of Hammar (1931) on the corresponding Hassall's corpuscles of the human thymus. He found that in conditions leading to a lymphocytosis, hyperplasia of the Hassall's corpuscles

occurred, followed after an interval by hyperplasia of the lymph node follicles and finally by the appearance of a lymphocytosis.

The original purpose of the present investigation was to determine the tissue producing the lymphocytosis stimulating factor found in the plasma of certain disease states—particularly chronic lymphatic leukaemia and lymphosarcoma.

The present work has shown that the thymus produces a lymphocytosis stimulating substance, both in normal and leukaemic animals and the evidence indicates the identity of thymic L.S.S. and the plasma factor. The features shown in common by the two are:

(*a*) production of a lymphocytosis in baby mice,
(*b*) inhibition of the development of a normal thymic L.S.S. content in inoculated mice,
(*c*) inhibition of activity by heating to 60° C.,
(*d*) inhibition of activity by the concurrent administration of cortisone or oestrogens.

Of these, the inhibition of the development of thymic L.S.S. is probably the most specific. Both plasma L.S.F. and thymic L.S.S. produce a lymphocytosis in inoculated mice whilst at the same time inhibiting the functional activity of the thymus in producing L.S.S. In the light of present knowledge of endocrine interrelationships, this finding can only indicate that both lie in a functional position between the originating endocrine gland and the target cell. Since one of the two, thymus L.S.S., is known to be produced by the thymus, then presumably both are.

The phenomenon of suppression of the thymic content of L.S.S. has enabled the lymphocytosis stimulating effects of thymus and thyroid emulsions to be differentiated.

Thyroid emulsions, although producing a lymphocytosis, do not inhibit the accumulation of L.S.S. in the thymus. It is probable, therefore, that the lymphocytosis in this case is produced by a stimulation of the mouse's own thymus to produce more L.S.S.

This concept is supported by Boyd's (1932) observation that a constant thymic hyperplasia is found in patients suffering from hyperthyroidism. The common clinical observation of a relative or absolute lymphocytosis in hyperthyroidism may find its explanation in these interrelationships.

From the above evidence for the close similarity between plasma L.S.F. and thymic L.S.S., it might be expected that the thymic content of L.S.S. would be raised in those diseases where L.S.F. was detectable in the plasma.

Preliminary observations on the thymuses of patients and mice with chronic lymphatic leukaemia have confirmed this expectation.

In the small series of thymuses examined, the thymic content of L.S.S. in the leukaemic animals has been consistently higher than the normal level. This finding needs confirmation with a larger series but at the moment it gives further support to the concept that plasma L.S.F. and thymic L.S.S. are identical.

The possibility remains that a deficient breakdown or elimination of thymus L.S.S. from the body may be responsible for the presence of detectable L.S.F. in the plasma and raised levels of L.S.S. in the thymus.

However, the finding of Miller and Turner (1943) of large amounts of lymphocyte stimulating material in the urine of patients with chronic lymphatic leukaemia makes this alternative unlikely.

There is thus good evidence for the presence of thymus overactivity in patients and mice with chronic lymphatic leukaemia and lymphosarcoma. What relationship has this finding to the disease states themselves?

Two possibilities seem to exist:

(*a*) the thymus overactivity represents a compensatory effort on the part of the body to maintain a normal complement of lymphocytic tissue in a situation where much of the pre-existing tissue has been rendered functionally inadequate by disease:

(*b*) the thymus overactivity may be aetiologically related to the disease states. Continued thymus overactivity may, by causing prolonged stimulation of the lymphocyte tissue, lead to a hyperplasia or eventual neoplasia of this tissue—either of which may constitute what is regarded as the leukaemic state.

There is little evidence to support the first hypothesis. However, several workers have produced evidence indicating that the second possibility may be correct.

Furth (1952), has shown that thymectomy reduces the incidence of lymphatic leukaemia in high-leukaemia strain mice. Similarly, Law and Miller (1950) and Kaplan(1950) have shown that thymectomy reduces the incidence of carcinogen- and radiation-induced leukaemia in mice.

Conversely, Law (1952) has shown that grafting of thymuses from high incidence strain mice to low incidence strain mice produces a definite elevation in the incidence of leukaemia in grafted mice.

Finally, it has been shown by Weymouth *et al.* (1955) that thymic tissue in irradiated mice contains an elevated level of RNA as compared with normal mice.

There is therefore considerable evidence to relate carcinogen- and radiation-induced leukemogenesis as well as genetically conditioned leukemogenesis with aberrations in thymic function. The evidence presented in this paper on thymic function in human and mouse leukaemia, indicates that thymic hyperactivity may represent the final common pathway by which the various leukemogenic factors induce the leukaemic state.

SUMMARY

1. Thymus and thyroid emulsions of human and mouse origin produce a lymphocytosis when injected into baby mice.

2. The thymus activity resides in the epithelial type cells.

3. Mouse thymus produces this lymphocytosis stimulating substance (L.S.S.) in tissue culture.

4. The content of L.S.S. in thymuses of humans and mice with chronic lymphatic leukaemia is much higher than normal.

5. Evidence has been presented that this thymic L.S.S. is identical with the lymphocytosis stimulating factor found in the plasma of certain disease states, particularly chronic lymphatic leukaemia and lymphosarcoma.

I am indebted to Sir Macfarlane Burnet, F.R.S. for his helpful advice and criticism throughout this work. I am also indebted to Dr. E. L. French for his advice and assistance with the tissue culture experiments.

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THE BONE MARROW IN MALIGNANT DISEASE

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MALIGNANT cells have been found in the bone marrow by many workers but have usually been considered to occur only in cases of advanced malignancy, and particularly in those in whom there is radiological evidence of bone metastases, (Stoger, 1941 ; Franke, 1942). In consequence sternal puncture has been only rarely advocated as a diagnostic procedure in malignant disease.

Recent advances in surgery and anaesthetics and the development of new techniques in radiotherapy and chemotherapy have made it important to assess patients who have a restricted primary growth from those with metastases. Sternal marrow examination by aspiration is now a simple procedure which may be undertaken without fear on out-patients and, in consequence, it was considered to be a possible method for searching for secondary spread.

Although Reich in 1935 reported the finding of carcinoma cells in bone marrow aspiration in one patient the first large study was that of Rohr and Hegglin (1936) who in a series of patients with advanced malignant disease found 10 positive aspirations among 74 examined. Since this time many reports have appeared in the literature of the finding of malignant cells. These findings have however been largely made on patients with advanced disease and of the 338 positive marrows reported in the papers only 40 were found in patients with negative radiological findings (Pillers, 1955).

Leitner (1949) has reviewed the main papers relating to this subject. In consequence further review here seems unnecessary. The present investigation was undertaken to find answers to the following problems.

1. What are the characteristics feature of malignant cells in the marrow and how may they be distinguished from other cells seen only rarely in marrow punctures.
2. Whether it is possible to find tumour cells in the sternal marrow in patients with clinically localised malignant tumours.
3. Whether there are any characteristic changes detectable in the bone marrow with malignant spread other than the finding of tumour cells.
4. To assess the value of sternal marrow examination in assessing the prognosis and deciding on the form of treatment to be given to patients with malignant disease.

MATERIAL AND METHODS

A group of patients was selected who were attending the Radiotherapeutic Department of Addenbrooke's Hospital for routine treatment, together with patients undergoing bronchoscopy at Papworth Hospital for suspected carcinoma of the bronchus and patients who had a radical mastectomy performed for carcinoma of

the breast. The exact selection of patients is considered in detail under the results but these groups included all types of malignant disease at all stages. The investigation was commenced in December, 1951, and all sternal punctures reported here were performed before August, 1955.

The clinical examination of the patient was made by the staff of the Radiotherapeutic Department and the authors were only informed of these clinical details after the marrow had been assessed. Thus it was hoped that bias in interpretation would be avoided. The follow up of the patients was done by seeing the patients personally or where they lived at a distance from Cambridge, studying the clinical records collected at the outside clinics attended by the radiotherapists. The follow up has been continued until March, 1956, and has varied from six months to just over four years.

The marrow aspirations were performed with a Turkel needle and the manubrium sterni was the site routinely chosen. Occasionally the marrow was aspirated from the body of the sternum when the presence of deformity or a previous fatty aspiration from the manubrium rendered it advisable. About 0·25 ml. of marrow fluid was withdrawn and after taking a few drops of the aspirate for 6–8 smears, the remainder was put into a Wintrobe's oxalate tube. From this tube any bone marrow flecks visible were picked by means of a fine wire loop. The fleck was spread on a glass slide. Both the film and fleck preparations were stained with Leishman stain. A total cell count was also performed on the oxalated marrow.

The film and fleck preparations were then initially examined individually and independently by two of the authors. A differential count of at least 200 cells was performed and in addition preparations thoroughly and systematically searched, the following points being noted :—

1. Cellularity.
   This was noted according to impression as low, normal or hypercellular. The classification corresponded almost exactly with the total counts recorded as under 20,000 ; 20,000–100,000 and over 100,000 respectively.
2. Plasma cell content.
   This was assessed on the general appearance including the finding of plasma cell nests, and on the differential marrow count.
3. The presence of abnormal cells in the marrow sample.
   As the films were examined microscopically cells that are not usually seen in marrow aspiration were encountered. These were then examined in further detail and an attempt made to define their exact nature. Some undoubtedly represented tumour cells while some were considered to be osteoclasts, osteoblasts or nests of degenerating plasma cells. The distinguishing features are considered later.

The two individual assessments were made entirely independently and the results then compared and the marrow classified into one of three groups namely:—

1. *Positive.*—The marrow contained cells considered to be definitely malignant.
2. *Suspicious.*—There was a definite plasmacytosis in the absence of any other cause with usually a hyperplasia of the other marrow elements. A few cases were included in this classification in which atypical cells were found but because they occurred in small numbers or singly could not be definitely classed as malignant cells. Such marrows, however, usually showed a plasmacytosis and would be classified as suspicious on that criteria as well.

3. *Negative.*—No abnormal cells and no abnormal bone marrow reactions were detected.

Only rarely has disagreement existed between the authors as to the classification and under these circumstances a compromise was reached as to the one recorded or if there was considerable doubt a repeat marrow examination has been made.

Marrow examinations have been made on 601 patients suffering from malignant disease. In addition to this, during this same time over 200 patients with diseases of the reticulo-endothelial system (e.g. leukaemia, Hodgkin's disease) had a marrow examination, but these patients are omitted entirely from discussion in this paper since marrow involvement may occur early in these diseases and the changes are already well recognised. Sarcomata were also excluded as the grade of malignancy is difficult to interpret. In addition, as a control, we have re-examined over 1,000 marrow punctures made during the past few years on patients with anaemia, on normal and abnormal infants (Gairdner, Marks and Roscoe, 1952) and many other generalised diseases including ankylosing spondylitis (Pillers and Marks, 1956). Among this control series no marrow which we would classify as positive was found.

Clinical assessment of the patient

The clinical stage of advancement of the malignant disease was recorded as early, intermediate or advanced according to the following criteria :—

1. *Early.*—Those patients with only apparently localised disease and with no detecable spread beyond the regional lymph nodes. This includes cases with mobile enlarged lymph nodes but excludes fixed nodes.

2. *Intermediate.*—Those patients showing clinical evidence of spread beyond (1) but no clinical evidence of involvement of the liver or clinical or radiological evidence of bone metastases.

3. *Advanced.*—Those patients in whom there is radiological evidence or clinical evidence of widespread metastases particularly in the bones or liver.

In addition to this assessment of the stage of advancement of the malignant disease patients were given a general medical examination. Most of the patients investigated were in good general health and in this present investigation much effort has been made to concentrate on the early cases rather merely examining patients already showing obvious evidence of widespread malignant disease.

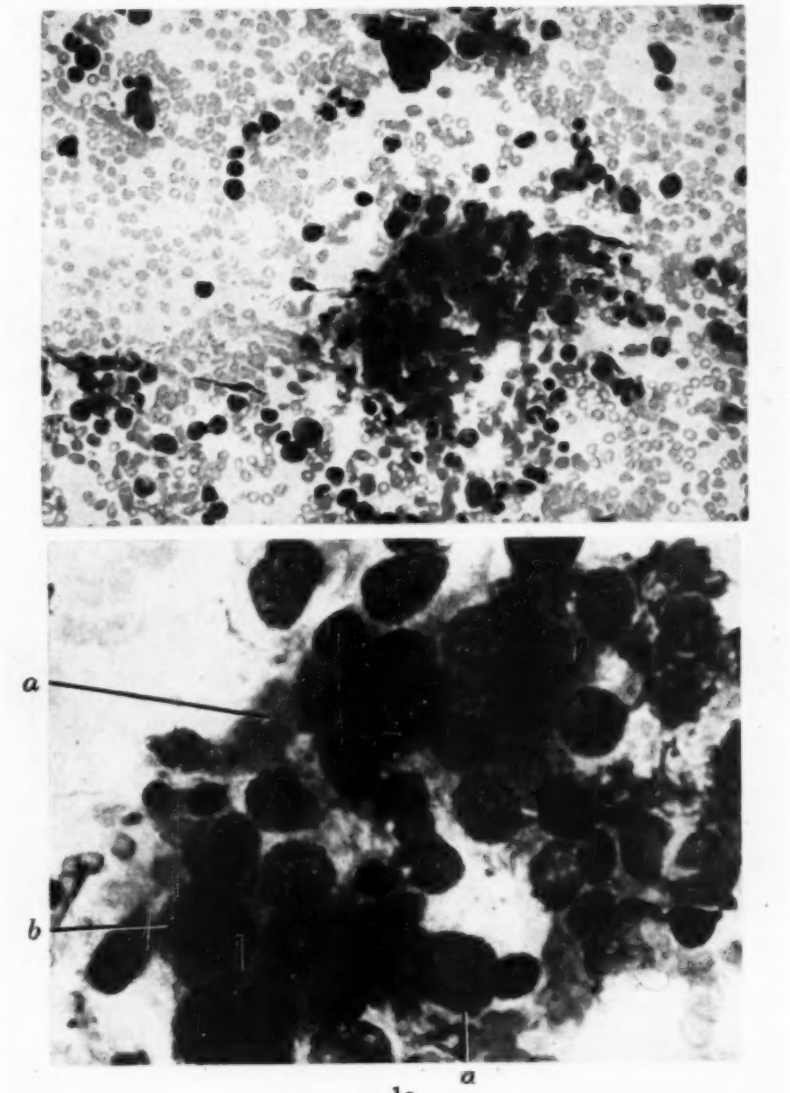
EXPLANATION OF PLATES.

FIG. 1*a*.—Low power (× 140) and high power (× 700) view of cluster of malignant cells. Note :—1. Grouped cells with syncytium (*a*) of lacy cytoplasm and indistinct borders. 2. Nuclei large, hyperchromatic, stippled chromatin and nucleoli (*b*).

FIG. 1*b*.—Group of osteoblasts (× 550). Note :—1. Eccentric nuclei. 2. Indistinct margin of cytoplasm (brilliant blue). 3. Archoplasm (*a*) may be at distance from nucleus. 4. Nucleoli (*b*).

FIG. 1*c*.—Osteoclast (× 550). Note :—1. Syncytial granulated cytoplasm (*a*). 2. Large round disconnected nuclei with stippled chromatin. 3. Nucleoli (*b*).

FIG. 1*d*.—Plasma cell " group " (× 550). Note :—1. Eccentric nucleus with dense chromatin. 2. Cytoplasm (dense blue) shows clear margin. 3. Perinuclear archoplasm (*a*).



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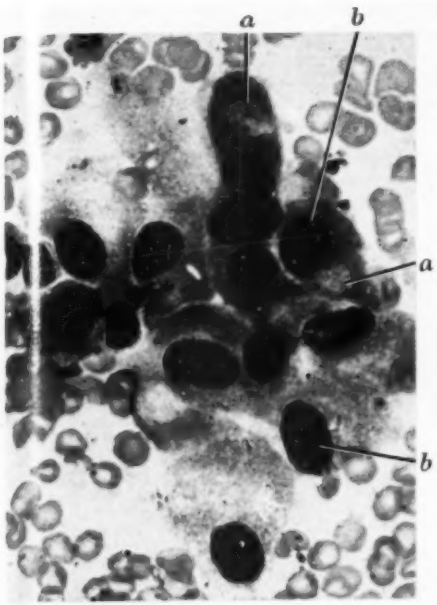

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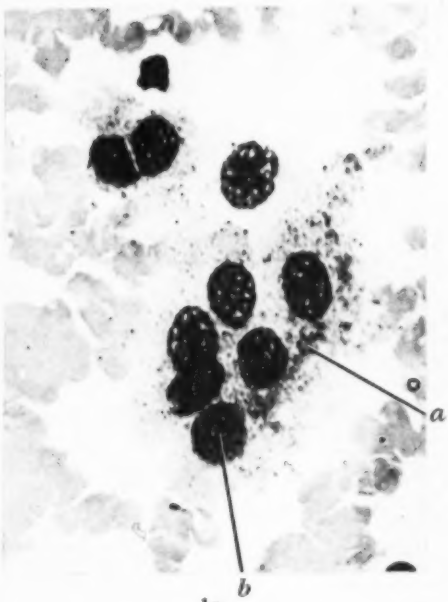

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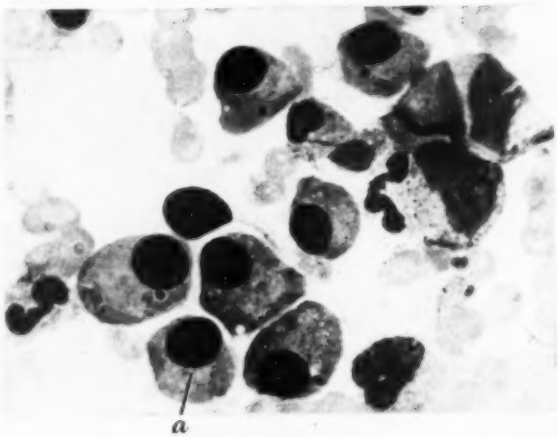

1d

The clinical staging we have used corresponds in the case of carcinoma of the cervix uteri to the following Stockholm staging. (Stockholm Report, 1951).

Early	= I + II
Intermediate	= III
Advanced	= IV

The survival times were calculated from date of marrow examination to the present time or to the date of death.

RESULTS

The present series may be divided into five main groups for consideration of results and the discussion, namely carcinomata of the breast, bronchus, cervix and ovary and a heterogenous group in which the individual types of tumours were too few to warrant separate detailed consideration. Details of the number and type of the cases that have been punctured and a summary of the marrow results are shown in Table I.

TABLE I.—*Details of the site of the primary tumour and the marrow results*

Site of neoplasm.	No. of patients studied.	Marrow results.		
		Positive.	Suspicious.	Negative.
Breast	190	12	42	136
Lung	125	14	47	64
Cervix	61	1	23	37
Ovary	30	2	14	14
Upper resp. tract	29	0	9	20
Alimentary canal	28	1	11	16
Male genital tract	20	0	2	18
Female genital tract	15	0	5	10
Urinary tract	17	0	7	10
Skin	20	1	4	15
Melanoma (all sites)	10	0	2	8
Endocrine system	11	0	4	7
Miscellaneous	19	0	3	16
Carcinomatosis (unknown primary)	26	6	7	13
Totals	601	37	180	384

Results in patients wth carcinoma of the breast

Excluding male patients and patients with a second primary neoplasm 190 patients with primary carcinoma of the breast have been examined.

The marrow results and survival times to date in this series are given in Fig. 2.

The survival times calculated to date for these patients show no obvious difference between the negative, suspicious or positive marrows although such results cannot be of great value at present in assessing the prognosis since 129 of the patients are still alive. The figures, however, may be considered in a different way, if evidence of progression of disease is taken as a criteria in each of the marrow groups.

Among the 136 patients with negative marrow 54 patients (40 per cent) are either dead or show evidence of disease progression. In the suspicious marrow group 26 patients (approximately 62 per cent) have progressed since the marrow

examination was made, while in the 12 patients with positive marrows 9 (75 per cent) of the patients show progression of the disease and 7 of these are dead.

Thus the results suggest that a further follow-up may result in a gradual difference in prognosis appearing between the three groups of patients since, in the negative group, there are far more patients who are now clinically and radiologically normal.

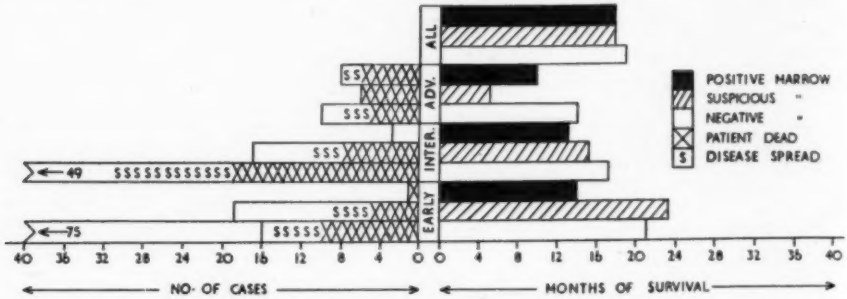

FIG. 2.—Results in 190 patients with carcinoma of the breast.

Results in patients with carcinoma of the bronchus

One hundred and twenty-five patients suffering from primary carcinoma of the bronchus have been examined. The diagnosis of carcinoma of the bronchus has been confirmed in these patients by thoracotomy, by biopsy, by bronchoscopy or in a very few cases only by the finding of malignant cells in pleural effusion or sputum.

The marrow results in this series and the survival times are given in Fig. 3.

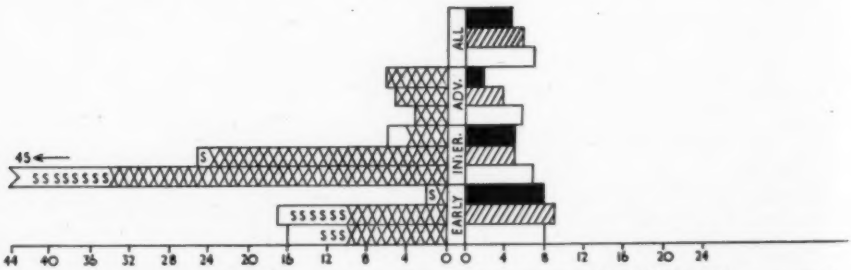

FIG. 3.—Results in 125 patients with carcinoma of the bronchus.

The division of these patients into stages is much more difficult than with other carcinomata. Early patients are those who show no extrathoracic spread and no involvement of the ribs and who on bronchoscopic evidence were considered suitable for surgery. Many of these patients subsequently underwent thoracotomy. The advanced cases were those who were, at the time of marrow examination, known to have liver or bone involvement, while the intermediate group showed

some extrathoracic spread but no clinical or radiological evidence of spread to the liver or bones.

The average survival times for the patients with negative marrows was seven months, for the suspicious marrows six months, and for the positive marrows five months. Since only nine of the patients are still alive with no evidence of spread of the disease these survival times are not likely to be greatly modified except for a possible slight prolongation of the group with negative marrows relative to the others.

Results in patients with carcinoma of the cervix uteri

The results of the marrow findings and the survival times for 61 patients with carcinoma of the cervix uteri who have been investigated are given in Fig. 4. Patients with negative marrow findings had an average survival time of sixteen

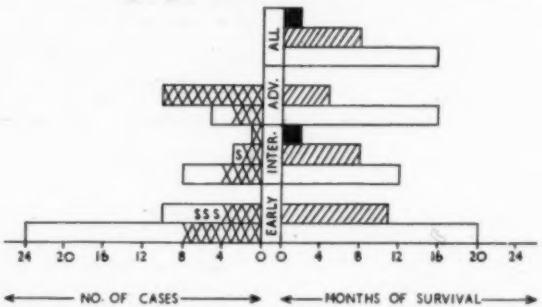

FIG. 4.—Results in 61 patients with carcinoma of the cervix.

months, those with suspicious marrows an average of eight months, while the patient with a positive marrow, even though classed as an intermediate case clinically died two months after the finding of a positive marrow. There is a statistically significant difference between the survivals in the negative and suspicious group. (Students t-test — $n = 32$; $t = 3{\cdot}36$; $P = 0{\cdot}002$; inclusion of the one positive marrow makes no difference to this significance.)

Among the patients with negative marrows 23 (63 per cent) are alive with no evidence of spread, while in the suspicious group only 3 (13 per cent) are still alive and well. Thus the difference between the negative and suspicious marrows should become considerably greater with a longer follow up.

The only positive marrow in this series showed no radiological evidence of spread and was, at the time of the marrow examination, in fairly good general condition. She died however two months later with clinical evidence of widespread disease.

Results in patients with tumours of the ovary

Thirty patients with ovarian tumours have been studied and the results are shown in Fig. 5. Classification into clinical stages has been done entirely on the findings at operation, early cases showed no evidence of spread outside the ovary and were operable. The intermediate group showed local extension and were

inoperable as far as radical surgery was concerned, while advanced cases showed liver and bone secondaries.

The average survival time in the patients with negative marrows was sixteen months, with suspicious marrows eleven months, and in two patients with positive marrows two months.

Both the positive marrow patients are dead, and only 2 (14 per cent) of the patients with suspicious marrows showed no evidence of further spread of disease.

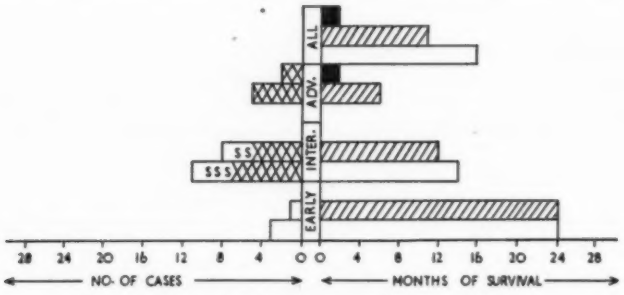

FIG. 5.—Results in 30 patients with ovarian tumours.

Among the patients with negative marrows 4 (28 per cent) are still alive and well. Thus in this group too, difference in survival times is likely to become more marked relative to the marrow classification rather than less marked. (Students t for all negative against positive and suspicious $n = 28$; $t = 2{\cdot}32$; $P = 0{\cdot}27$ and is significant at the 5 per cent level.)

Results in a group of patients with carcinoma of various sites and types

One hundred and ninety-five patients suffering from primary neoplasms of sites other than those already mentioned have been investigated. The diagnosis and the marrow findings are given in Table I. In a mixed group of this sort with diseases having varying natural histories follow up data would be valueless. Positive marrow aspirations were found only in two patients in whom the site of the primary growth was known (stomach and skin), but six patients with carcinomatosis with an unknown primary tumour have yielded a positive aspiration. Suspicious marrows were found only in 3 patients.

The marrows eosinophil count in patients with malignant disease

A total eosinophil percentage in the marrow has been calculated for each puncture. Table II gives details of the patients of the four main groups who showed a marrow eosinophilia (over 7·5 per cent). Eighteen out of 406 patients show a marrow eosinophilia but there is no apparent correlation in the clinical status or the follow up of these patients.

No eosinophilia was found among the patients with positive marrows and the incidence of eosinophilia among the patients with suspicious marrows was not more than would be expected by chance if the frequency of each of these conditions is considered.

TABLE II.—*Clinical staging, marrow result, and actual and calculated follow-up on patients showing a marrow eosinophilia*

Diagnosis.	Case No.	Clinical status.	Marrow result.	Follow-up.	Expected follow-up.
Carcinoma, breast	33	E	— ve	33 spread	21
	77	E	— ve	7 dead*	21
	79	A	sus	7 dead	5
Carcinoma, bronchus	2	I	sus	3 dead*	5
	10	I	sus	10 dead	5
	11	E	sus	11 dead	9
	26	I	— ve	2 dead*	7
	28	E	sus	4 dead*	9
	60	I	— ve	2 dead*	7
	92	I	— ve	11 dead	7
Carcinoma, cervix	7	I	— ve	38 a. & w.	12
	17	E	— ve	29 „ „ „	20
	20	E	sus	3 dead*	11
	33	I	— ve	20 a. & w.	12
	40	I	sus	2 dead*	8
Ovarian tumours	1	I	sus	12 dead	12
	4	I	— ve	18 dead	14
	12	I	— ve	12 dead*	14

* Patient with a poorer prognosis than expected.

DISCUSSION

The identification of malignant cells in the marrow

The identification of malignant cells in the marrow is often a matter of considerable difficulty, and requires knowledge of the normal marrow cells under a variety of conditions.

Since the primary tumours differ so much in their morphology it is only to be expected that the nodules in the marrow would show a similar considerable variation in cytology. Thus it is only possible to give broad generalisations of the distinguishing features. These features can be summarised as :—

1. The general sturcture and staining qualities of the cells are at variance with those of the normal cells of the marrow, i.e. the cells are foreign to the normal marrow elements.

2. The cells are usually arranged in a group of cluster and have a tendency to form a syncytium.

3. There is a great variation in the cell size, but the cells are usually large—10 to 30μ in diameter.

4. The cytoplasm may be abundant or scanty. When abundant it is often fragile, lacy or vacuolated with indistinct borders and may even be absent. It may be slightly or deeply basophilic in staining.

5. The nucleus is usually large and hyperchromatic with stippled nuclear chromatin.

6. The nucleoli are usually prominent, usually 1–3 in number and may be disproportionately large in relation to the nucleus. As described by Stoger (1941) the smaller cells may have no nucleoli, but the finding of these small malignant cells with no nucleoli is extremely rare and the authors feel that considerable

care should be exercised in identifying these as true tumour cells. The majority of such cells are probably plasma cells.

7. Many other workers describe mitotic figures as being numerous, but in our experience they appear to be numerous only in those cases in which the marrow is entirely replaced by sheets of malignant cells.

At the present stage of the investigation it is not considered possible to determine the site of the primary tumour from the cytological characteristics of the cells found at bone marrow puncture. Rohr and Hegglin (1936) suggested that small cells occurring in groups might indicate a secondary from a carcinoma of the bronchus while large cells sometimes occurring singly could suggest the prostate or stomach as primary. Amy and Jaimet (1953) reported similar findings. Rohr's figures are however on the basis of 7 positive aspirations in carcinoma of the bronchus and in intestinal carcinoma. In the present work we have been unable to confirm their conclusions in carcinoma of the bronchus for out of the 14 positive aspirations that we report only 2 showed clusters of small cells. There are certain other cells which may easily be confused with malignant cells in the marrow. Some of these have undoubtedly confused other authors to judge from the photomicrographs that they reproduce and have caused us considerable worry at times in arriving at a firm conclusion.

Fig. 1*a* shows a typical cluster of malignant cells.

Osteoblasts and osteoclasts

These cells would seem to produce more confusion than the rest. They were described by Esser in 1945 and were stated by her to occur much more commonly in children's marrows than adult. Although these cells are normal in bone biopsies they are seen only rarely in aspiration samples of marrow. They may occur either singly or more commonly in our experience in small clusters.

The osteoblasts closely resemble plasma cells with their eccentric nuclei and brilliant blue cytoplasm, but are much larger in size—20–30μ. They may show azurophilic granules, and the margin is usually indistinct. The pale round area of archoplasm is some distance from the nucleus whereas it is adjacent to the nucleus in plasma cells. There are usually one or two nucleoli.

The osteoclasts are multinucleate cells with big round disconnected nuclei, and granular cytoplasm which shows some resemblance to the cytoplasm of megakaryocytes.

These cells have been stated (Tischendorf and Heckner, 1950) to be present in the marrow only under certain pathological conditions in adults but we have seen them both singly and in clumps not only in carcinoma, but also in Hodgkin's disease, spondylitis and other non-malignant conditions. As far as we can determine they have no especial significance relative to the conditions, but may provide diagnostic difficulty.

Fig. 1*b* and 1*c* show typical osteoblasts and osteoclasts in sternal marrow aspiration.

Plasma cell nests and degenerating plasma cells

Degenerating plasma cells may occur either singly or in clumps, and are larger than most other normal marrow cells. The nucleus may show pyknosis with early fragmentation and occasionally an intra-nuclear vacuole which may give the

appearance of a false nucleolus. The cytoplasm is plentiful and usually faintly staining, while the cell outline is often indistinct. These cells are described by Hayhoe and Smith (1951) and occur in many conditions other than malignant disease.

Plasma cells commonly occur singly in the marrow but where there is a plasmacytosis they may also be found in nests. Frequently these nests contain a multinucleate plasma cell in addition to several with single nuclei, and the first impression is often of a syncytium. The dense blue cytoplasm, the dense chromatin in the nucleus with no nucleoli and the perinuclear archoplasm, distinguish these nests from a cluster of malignant cells.

A typical plasma cell group is shown in Fig. 1*d*.

The incidence of positive aspirations

Among the present series there has been an incidence of 6·2 per cent positive aspirations (37 out of 601 patients) or 7 per cent in the four main groups considered (29 out of 406 patients). This at first sight compares very unfavourably with the results of previous workers (Table III), in patients with similar diagnoses. Thus overall they found approximately 32 per cent of the patients had positive marrow findings. This is to a very considerable extent due to the different selection of patients. Among the patients with positive aspirations in the four main groups of this present series 18 or 62 per cent were found in patients with no radiological evidence of spread of disease. In the patients investigated by other workers on the other hand only 10 per cent of the positive aspirations were found among patients with no radiological evidence of spread. Previous workers have in fact almost entirely confined their attentions to patients with advanced disease. The present series on the other hand deliberately concentrated on the less advanced cases.

TABLE III.—*Comparison of the positive marrow aspirations in the present series with those reported by previous workers in patients with primary tumours of similar sites*

Author.	Breast.	Lung.	Cervix.	Ovary.	Total.
Vogel, Erf and Rosenthal 1937	2 (2)	0 (5)	0 (1)	—	2 (8)
Kryeberg and Poppe, 1940	3 (26)	0 (1)	0 (1)	—	3 (28)
Selberg, 1942	3 (6)	4 (12)	.	—	7 (18)
Franke, 1942	2 (11)	5 (22)	2 (5)	0 (2)	9 (40)
Gormsen, 1942	19 (98)	2 (7)	—	—	21 (105)
Weisberger and Heinle, 1948	2 (10)	2 (8)	0 (2)	—	4 (20)
Lanier, 1949	2 (5)	0 (2)	0 (2)	—	2 (9)
Jonsson and Rundles, 1951	8 (14)	6 (14)	—	—	14 (28)
Rubinstein and Smellin, 1951	21 (35)	4 (10)	—	—	25 (45)
Rubinstein and Smellin, 1952	20 (32)	5 (10)	—	—	25 (47)
Total	82 (239)	28 (96)	2 (11)	0 (2)	112 (348)
Present series	12 (190)	14 (125)	1 (61)	2 (30)	29 (406)
	4*	12*	1*	1*	18*

* Denotes the number of patients showing positive marrow aspiration and no X-ray evidence of bone metastases.

Sternal aspiration as a method for the detection of tumour cells

In order to find tumour cells in the maximum number of patients one should puncture the bone that shows radiological evidence of spread. We have, on occasions, taken radiographs of various bones including the sternum to find a suitable site for puncture but such radiographic examinations are costly and time consuming and are not feasible in many units undertaking large numbers of routine examinations. In addition it has already been shown that positive aspirations may be found where there is no radiological evidence of disease.

Bone marrow aspiration can be performed in the sternum, the iliac crest and the vertebrae. There is evidence both from autopsy examinations (Selberg, 1942) and from patients with multiple examinations (Clifton, Philip and Fowler, 1952) that sternal marrow aspiration yields a slightly higher number of positive results than either iliac crest or vertebral spine examinations where previous radiographic investigation is not undertaken. The finding of cells in the sternal marrow cannot be ascribed to direct extension in carcinoma of the breast and bronchus.

Thus where it is impossible to determine the site of secondaries by radiographic examination or where there is no evidence of spread, examination of the sternum is probably the method of choice and is the one that we have adopted.

The significance of a marrow plasmacytosis

An increase of plasma cells in the marrow has been reported in a variety of conditions both physiological and pathological. Among these may be mentioned rheumatoid arthritis (Hayhoe and Smith, 1951). chronic infections, measles, cirrhosis, and Hodgkin's disease ; for review of cause of marrow plasmacytosis *vide* Fadem and McBirnie (1950).

Such an increase has however been reported to occur in malignant disease and in such cases may indicate the presence of a generalised spread. Stoger (1941) examined the marrows of 110 patients with malignant disease and found that in 84 per cent of his tumour free biopsies a plasma cell reaction from 1·1–16 per cent plasma cells was present. Morel (1947) suggested that a plasmacytosis greater than 4 per cent suggested the presence of a widespread neoplasm, while Lanier (1949) in her series of 32 cases found numerous clumps of plasma cells. Amy and Jaimet (1953) similarly found an increase of plasma cells from 2 to 13 per cent.

In view of the numerous conditions that can give rise to a plasmacytosis this reaction can in no way be considered specific for malignancy. Such a reaction should be considered as evidence of some generalised body disturbance and a cause sought. In the absence of other cause in a patient with an already proven malignancy it is possible that the presence of this neoplasm may be the exciting factor.

In the present series those patients with a plasmacytosis have been fully investigated in an attempt to discover any other possible cause. When none has been found they have been included in the classification of patients with a suspicious marrow. The significance of this plasmacytosis in relation to the prognosis of patients with malignant disease is considered in a subsequent section.

The significance of a bone marrow eosinophilia

A few previous workers have noted an eosinophilia in the bone marrow of patients with neoplastic disease. Morel (1947) felt an increase of eosinophils of over 5 per cent was significant of neoplasm where no other cause could be found.

Lanier (1949) found an increase in 44 per cent of her patients but cited Nordensen (1935) as stating that it was an infrequent occurrence in his series of 17 patients.

In the present series 18 out of the 406 patients analysed showed an eosinophilia (over 7·5 per cent mature eosinophil and precursors). There is at present no apparent correlation between the clinical status and the follow up of these patients. No eosinophilia was detected in the 37 patients with positive bone marrow aspirations. Eight of the 18 patients with eosinophilia were classified by other criteria as suspicious.

Among the patients with a marrow classification of suspicious and an eosinophilia 4 (50 per cent) had a prognosis poorer than the average expected survival time, among those with eosinophilia and negative marrows 4 (40 per cent) had a prognosis poorer than the average expected survival time.

Thus at the present time there is little evidence that eosinophilia can be considered to signify poor prognosis in malignant disease.

The relationship between the marrow classification and the clinical follow up

The four main groups considered have an entirely different natural history and therefore the results of the follow up study must be considered separately for each type of primary neoplasm.

In carcinoma of the bronchus patients showing a positive marrow have an average survival time of five months, those with a suspicious marrow six months and those with a negative marrow seven months with a greater percentage of the patients still alive and well who had a negative marrow examination. Thus the prognosis when a positive marrow was found seems to be rather shorter than with either a suspicious or a negative marrow while a suspicious marrow gives a slightly poorer prognosis than the negative marrow. Differences in the average survival times however are short.

In carcinoma of the cervix average survival times are significantly longer with a negative marrow than with either a suspicious marrow or a positive marrow, and the patient with a positive marrow showed an extremely short survival time. These differences are likely to become more pronounced for the majority of the patients with a negative marrow are still alive and well.

In ovarian tumours two patients with a positive marrow showed a very short survival time while those patients who had a negative marrow had a slightly better prognosis than those with a suspicious marrow.

In carcinoma of the breast no differences can be found so far in the series examined between the average survival times of patients with negative, suspicious and positive marrows but in carcinoma of the breast the overall survival time is long and the majority of patients are still alive and well. If evidence of spread of the disease is considered, however, more patients with positive and suspicious marrows show evidence of spread. A longer follow up however will be necessary before clear-cut results can be found for carcinoma of the breast.

Considering each of these groups there is evidence to suggest that correlation exists between the marrow findings and the clinical follow up. Marrow puncture can give evidence of spread of the disease but although this would on the average signify a poor prognosis it does not of course necessarily signify a very early death and should be used as a further indication of general spread.

The finding of a suspicious marrow on the other hand cannot be considered as pathognomonic of malignant spread for many other conditions can give these

changes. A suspicious marrow however must not be dismissed until a cause has been found. Should no other cause be determined, there is evidence from this present study to suggest that tumour spread may exist with a consequent poor prognosis to the patient.

CONCLUSIONS

The value of sternal puncture in the treatment of carcinona

The present results in this series suggest that the following firm conclusions may be drawn.

1. Sternal puncture has a definite value in assessing the prognosis in both carcinoma of the cervix and carcinoma of the ovary but has little value in determining the prognosis in carcinoma of the bronchus. In carcinoma of the breast the natural history of the disease is so long that no definite conclusions can be drawn at present as to the value of sternal puncture in assessing prognosis.

2. Sternal puncture has a definite place in assessing the type of treatment that should be used.

(*a*). Carcinoma of the bronchus.

Where there is evidence of spread as determined by a positive marrow aspiration major surgery should be avoided except as a purely palliative measure.

(*b*) Carcinoma of the ovary.

Here a decision must be made between palliative and radical X-ray therapy. With a negative marrow examination there is strong evidence from this series that no spread exists and under these circumstances radical therapy in the maximum tolerated dose is indicated.

(*c*) Carcinoma of the cervix.

Those patients who show a suspicious or positive marrow and fail to respond locally to primary radiotherapy have not benefited greatly from Wertheim's radical abdominal hysterectomy.

3. It is possible by sternal marrow examination to detect spread of tumour earlier than with X-rays but the method is extremely time-consuming (about three hours per patient) and requires considerable experience of normal and abnormal bone marrows. It consequently will probably have no place in the routine radiotherapeutic department.

4. Sternal marrow may provide cytological material for diagnosis but at the present moment only of the presence of malignancy. With further experience the site of the primary tumour may possibly be diagnosed.

5. The finding of a suspicious marrow should be used as a guide to the presence of organic disease, and a suspicious marrow should never be dismissed until the cause is determined.

SUMMARY

The results are reported on sternal marrow examination of 601 patients suffering from carcinoma. The majority of these patients were clinically well and at an early stage of the disease. Four hundred and six patients with carcinoma of the breast, bronchus, cervix uteri and ovary are considered in detail.

The follow-up has varied from six months to over 4 years.

Thirty-seven patients showed tumour cells in the marrow, while 180 showed a marrow plasmacytosis. Evidence is presented that prognosis is worst with tumour

cells in the marrow, but that in carcinoma of the bronchus, cervix uteri and ovary, a marrow plasmacytosis is associated with a poorer average survival than patients showing no marrow plasmacytosis.

It is suggested that sternal marrow examination has a place in assessing the type of treatment and in providing cytological material for diagnosis of malignancy.

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HUMAN TUMOURS IN CORTISONE-TREATED MICE*

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IT is the aim of the present article to contribute to the study of conditions for transplantation of human malignant tumours to mice.

Already from studies by Loeb (1901) and C. O. Jensen (1902, 1903) it appeared that heredity and immunity would play an important part in the fate of tumour grafts, and this view was further elaborated by Murphy (1913), Tyzzer (1916*a*, 1916*b*) and Bittner (1931). According to these authors, the more foreign the cells the more powerful they are as antigens, and the so-called "secondary immunity" would therefore be expected to be pronounced when human tumour cells are transferred to animals. Consequently, it seemed necessary to avoid or at least to reduce the immunitory response in experimental animals, if attempts at heterologous transplantation should have any chance of success.

Some workers have chosen to transplant into newborn animals (Gheorghiu, 1926) with only feeble immunitory response, others to the anterior chamber of the eye (Keysser, 1913; Smirnova, 1937; Greene, 1938, 1941, 1947, 1948, 1950; Greene and Lund, 1944; Towbin, 1951*a*), and others to the brain (Greene, 1951; Albrink and Greene, 1953) in which organs no such response seems to be released.

In order to facilitate heterologous transplantation, universal roentgen irradiation was introduced by Murphy (1914), and later elaborated by Krebs and associates (Wagner, 1930; Krebs and Busch, 1931; Clemmesen, 1940; Bichel and Holm-Jensen, 1949) using heavy doses before subcutaneous or intramuscular transplantations as a means of eliminating the formation of antibodies. However, roentgen irradiation is inefficient in having only temporary effect, and is particularly unsuited for the transmission of human tumours (Toolan, 1951), which will be followed by a relatively long latent period before an eventual take (Towbin, 1951*b*). Furthermore, a second irradiation might interfere with the life of the transplanted cells.

On the contrary, cortisone in heavy daily doses will restrain the function of antibody-producing centres without significant damage to the graft. Only tumours of lymphoid origin will, like normal lymphoid tissue, be considerably hampered by heavy doses of the hormone (Pearson, Eliel and Rawson, 1950; Spies, Stone, Lopez, Milanes, Toca and Reboredo, 1950; Kirschbaum, 1951; Block and Jacobson, 1952).

Thus, Green and Whiteley (1952) found that massive doses of cortisone would considerably prolong the survival of human tumours transplanted to experimental animals, and these observations were confirmed by Toolan (1953), who later (1954) succeeded in keeping three human squamous cell carcinomas and two human sarcomas alive through alternate passages in rats treated with cortisone and in

* Extension of the paper read at the 20th congress of the Scandinavian Association for Medical Radiology in Gothenburg, June 1955.

the cheek pouch of hamsters. Patterson, Chute and Sommers (1954) similarly succeeded in transmitting a human squamous cell carcinoma through many passages in the cheek pouch of hamsters treated with cortisone. It appeared from these publications that a few out of a series of tumours tested showed capacity of growth in the heterologous host, but only when the production of antibodies was suppressed with cortisone.

Author's Experiments.—In the experiments reported in the following the author has exploited the possibilities for heterologous transplantation studies offered at the Finsen Institute and Radium Centre in Copenhagen. Here a large number of patients with malignant diseases are concentrated close to experimental laboratories.

MATERIAL AND METHODS

Experimental animals were mice belonging to a subline of the so-called Bagg strain, which shows a low incidence of spontaneous tumours, according to Engelbreth-Holm (1956, personal communication) 5 per cent out of 343 animals. Only one mouse under ten months of age developed a spontaneous tumour (leucemia). At transplantation, animals weighed 17 to 20 grams, and were 2 to 3 months old. Equal numbers of each sex were entered into experimental and control groups.

Cortisone treatment began at least four days before transplantation, and continued with a daily dose of 1 mg. until the animal died or was killed. In order to keep the dosage of cortisone precise, the employed solution of hormone, "Cortone Merck", was diluted up to a concentration of 1 mg. cortisone in 0·1 ml. of the solution. Control animals had daily injections of 0·1 ml. physiological NaCl. In order to obtain an universal effect, the hormone was injected intraperitoneally at subcutaneous or intra-ocular transplantations, and subcutaneously at intraperitoneal inoculations.

In a few exceptional series it was attempted to increase the effect of the hormone by universal roentgen irradiation 24 hours before transplantations (dosage: 200 r; intensity: 26·4 r/min.; HVL: 1·0 mm. Cu; 180 kilovolt; 6 milliamp.; 0·5 mm. Cu-filter).

Tumours were minced with scissors and injected into animals by means of a syringe with a screw-driven piston. At subcutaneous and intraperitoneal inoculations the transplant amounted to 0·05 ml. of the mince, while ascites fluid was injected in quantities of 0·5 ml. on account of the relatively smaller contents of tumour cells. The intra-ocular transplantations were carried out on ether anaesthetized animals. Through a cut at the upper cornea-scleral junction a butt canula was introduced, containing tumour mince, which could be pushed out by means of a well-fitting mandrin. On account of the small dimensions of the mouse eye, the application of the graft was not always quite successful.

All transplantations were undertaken alternately to cortisone-treated and control animals. Instruments were sterilized in boiling water for 20 minutes.

As a rule, human tumours used were metastases or local recurrences, which were supposed to have the greatest possibilities of taking. Transplantation material was removed from patients during operation under general anaesthesia, and immediately brought to the laboratory. Usually 15 or 20, but in no case more than 30 minutes elapsed from the cessation of blood supply to the tissue in question

until all animals had been transplanted. Inoculation of ascites fluid was always finished within 5 min. after puncture.

In the transplantation series where tumour mince was used, animals were killed at fixed intervals, usually with one mouse from each group, starting at the tenth day after transplantation and subsequently every fifth day. Mice inoculated with ascites fluid were not killed, but examined by punctures, usually every tenth day.

RESULTS

Transplantation results with 21 different human tumours are shown in Tables I and II. It appears that in the cortisone-treated groups of 12 experimental series, tumour tissue apparently capable of living was found at the site of inoculation after the tenth day in at least one animal, while the grafts in control mice had completely disappeared, leaving a dense infiltration of lymphocytes and granulocytes. Such foreign-body reaction was never found in animals treated with cortisone.

In the series where tumour mince had been used for transplantation, tumour tissue was demonstrated up to 45 days later, but without proliferation or invasiveness, though it appeared capable of living and had kept its characteristics (Fig. 1 and 2).

No difference between the results of subcutaneous and intraperitoneal application of the graft could be distinguished, and adaptation of the tumours to cortisone-treated animals was not furthered by roentgen irradiation or by transplantation to the anterior chamber of the eye.

In one of the two cases in which inoculation of human ascites fluid into the peritoneum of mice was attempted, it resulted in the formation of an ascites tumour which has now been transmitted through 41 serial passages in mice of Bagg's strain (March 1956). The ascites fluid in this case was particularly rich in cells, and presented some other features worth mentioning.

The Ascites Tumour H.A.I

Case history.—In brief the case history of the patient was the following: Female aged 75, born 1879 (R. No. 64561). Syphilis was ascertained 1930, but never treated. Since 1934 liver function had been failing as indicated by formation of ascites and positive results from liver function tests. In 1939 an adenocarcinoma developed in the right breast with metastases to axillary lymph nodes (Fig. 3). Radical operation was attempted but presumably without full success. No radiation treatment. Roentgenographic changes in spinal column and pelvis were suspicious of malignancy, but no proof was attained. In 1952 a solid carcinoma of the cervix (Stage III) (Fig. 4) was treated with roentgen irradiation and radium, but cachexia developed accompanied by increasing ascites which had to be drained every second week. Cytological examination of ascites smears showed large numbers of cells differing in size, in number of nuclei and showing many mitoses (Fig. 5), but without tissue characteristics. Death occurred in March 1955, and autopsy showed metastases from a squamous cell carcinoma in abdominal lymph nodes, peritoneum and peritoneal fat. The osseous changes mentioned above were not verified at autopsy.

TABLE I.—*List of Human Tumours Transferred to Mice Treated with Cortisone, Cortisone + Roentgen Irradiation or Roentgen Irradiation alone, Tumours Surviving 10 days or more.*

(The parentheses in the result columns indicate the longest period [in days] after which tumour tissue was found in the groups concerned.)

Patient No.	Sex.	Tumour.	Source of graft.	Application.	Number of mice with takes. Cort.	Cort.+Rtg.	Rtg.	Control.
Z.1519	f.	Adenocarcinoma (Oesophagus)	Skin metastasis	Subcutaneous	3/9 (45)			0/10
Z.1870	m.	Squamous cell carcinoma (Oesophagus)	Lymph node metastasis	Intraperitoneal	5/8 (30)			0/9
Z.1875	m.	Squamous cell carcinoma (Oesophagus)	do.	Subcutaneous	1/5 (15)			0/5
				Intraperitoneal	1/5 (20)			0/5
Z.1803	f.	Adenocarcinoma (Stomach)	Liver metastasis	Subcutaneous	1/8 (15)			0/10
Z.1915	m.	Adenocarcinoma (Stomach)	Lymph node metastasis	,,	2/9 (35)			0/10
				Intraocular	0/5			0/5
Z.1858	f.	Adenocarcinoma (Breast)	Primary site	Subcutaneous	2/10 (35)	4/8 (30)	0/10	0/10
Z.1878	f.	Adenocarcinoma (Breast)	,, ,,	Intraperitoneal	2/9 (20)	0/8	0/10	0/10
Z.1896	f.	Solid carcinoma (Breast)	,, ,,	Intraperitoneal	1/10 (20)			0/10
				Intra-ocular	0/5			0/5
Z,1628	m.	Malignant melanoma	Lymph node metastasis	Subcutaneous	1/5 (15)			0/5
				Intraperitoneal	4/5 (40)			0/5
Z.1913	m.	do.	do.	do.	0/5			0/5
					2/4 (20)			0/5
Z.1855	m.	Fibrosarcoma (Back)	Primary site	,,	0/5			0/5
					1/5 (10)			0/5
R.64561	f.	Squamous cell carcinoma (Cervix) Solid carcinoma (Breast)	Ascites	Intraperitoneal	4/9 (Permanent growth)			0/10

TABLE II.—*List of Human Tumours Transferred to Mice Treated with Cortisone, Cortisone + Roentgen Irradiation or Roentgen Irradiation alone, but regressing within 10 days in both Treated and Untreated Mice.*

Patient No.	Sex.	Tumour.	Source of graft.	Application.	Number of mice			
					Cort.	Cort. +Rtg.	Rtg.	Control
Z.1826	m.	Adenocarcinoma (Stomach)	Lymph node metastasis	Subcutaneous	10	—	—	10
Z.1900	m.	Adenocarcinoma (Stomach)	Skin metastasis	Intraperitoneal	3	—	—	5
				Subcutaneous	5	—	—	5
Z.2013	f.	do.	Liver metastasis	do.	5	4	5	5
					4	5	5	5
Z.1843	m.	Malignant melanoma	Primary site	Intraperitoneal	9	—	—	9
Z.1783	f.	do.	Lymph node metastasis	Intraperitoneal	5	—	—	5
				Subcutaneous	10	—	—	9
Z.1937	f.	Hodgkin's disease	Spleen	do.	5	—		5
					4	—	—	5
Z.1818	f.	Solid carcinoma (Bladder)	Primary site	Intraperitoneal	8	—	—	10
				Intraocular	5	—	—	5
R.74760	m.	Adenocarcinoma (Prostate)	Testis metastasis	Subcutaneous	10	9	9	10
R.78083	f.	Adenocarcinoma (Ovary)	Ascites	Intraperitoneal	10	8	—	10

Transmission to mice.—Ascites fluid recovered seven months before death, and immediately injected into peritoneum of ten mice treated with cortisone, and ten controls, caused no immediate response except the death of one experimental animal on the day of inoculation.

Twenty-eight days later two female and one male mouse had developed marked distention of the abdomen, which increased rapidly so that all these animals were unmistakably distended from about the 45th day (Fig. 6). In all three cases punctures on the 25th day resulted in yellowish, slightly turbid fluid containing numerous tumour cells, closely similar to the patient's although perhaps varying more in size (Fig. 7). Some of them showed pronounced vacuolation. Cells from the mice had no admixture of granulocytes, which was pronounced in the human ascites.

A fourth experimental female mouse developed increase in abdominal size following the 52nd day, and gave the same results from cytological examination of ascites as already described.

*Serial transmission of the ascites tumour H.A.*1

On the 35th day ascitic fluid from each of two females out of the first three positives was transmitted into five cortisone-treated and five control animals. The twenty animals of this second passage each received 0·1 ml.

As shown in Table III, all of the ten cortisone-treated animals developed ascites, observable from the 20th day, from which date it increased rapidly until death occurred, on the average 56 days after inoculation. Repeated punctures gave fluid with a cytological picture corresponding to the description given above. It should be noted that there was complete identity in picture between fluids from the two groups of animals derived from each of the two mice in the first passage.

Ascites developed in none of the control mice from the first two passages, and at autopsy three months after inoculation neither controls nor negative cortisone-treated animals showed any abnormal findings.

It is worthy of particular notice that the animals from the first two passages in which inoculation caused ascites, showed nothing abnormal at autopsy except the ascitic fluid amounting to almost 8 grams on the average (Table III). Histological examination of the peritoneum and abdominal organs showed no pathological change.

TABLE III.—*The Ascites Tumour H.A.*1. *in the First Passages in Mice.*

Passage No.	Treatment.	Number of mice with ascites.	Average lifetime in days.	Average amount of ascites in grams.
1	Cortisone	4/9	66·5 (53–88)	7·9
	Controls	0/10	—	—
2	Cortisone	10/10	56·0 (46–63)	7·7
	Controls	0/10	—	—
3	Cortisone	9/9	32·4 (25–41)	8·6
	Controls	10/10	36·2 (24–50)	6·4
4	Cortisone	10/10	30·9 (24–38)	7·4
	Controls	10/10	28·7 (19–40)	7·1

The third passage originated from ascitic fluid recovered from the previous passage 35 days after transmission, and inoculated into ten cortisone-treated animals and ten controls. In this third passage, however, the formation of ascites occurred also in the untreated controls, abdominal distention being noticeable in treated and untreated animals between the tenth and fifteenth day. Henceforth, the abdominal increase developed considerably more rapidly than in the previous two passages, and the lifetime of the animals was correspondingly shortened in both groups (Table III).

A fourth transmission was carried out on both cortisone-treated and untreated mice, but as the ascites tumour now seemed to grow equally well in the two groups, serial transmission was continued in normal mice only.

The tumour has up to now (March 1956) been propagated through 41 passages, each comprising ten mice, five of each sex. The inoculation dose has been kept at 0·1 ml., and the ascitic fluid recovered at autopsy usually amounts to between seven and ten grams.

From the third passage inoculation has not failed to cause ascites tumour in any case, and development of the latter has taken place with increasing rapidity, incipient distention now appearing 3 to 4 days after inoculation. In the last ten passages the average life time after inoculation has been 18 days for males and 15 days for females.

While autopsy in the first two passages did not disclose any solid tumours or metastases, all mice in the third and following passages have shown extensive semi-solid and solid tumour growths (Fig. 8), often with infiltration of intestine, stomach, pancreas, liver, kidneys and lymph nodes (Fig. 9 and 10). Distant metastases were not observed.

DISCUSSION

The favourable effect of cortisone on inoculates of human tumour cells in mice may be attributable to a depressing action of the hormone on the production of

antibodies (Stoerk and Solotorovsky, 1950; Bjørneboe, Fischel and Stoerk, 1951; Kass, Kendrick and Finland, 1953). A further effect may be due to a decrease in the number of macrophages (Baker, 1952) together with a reduction in their phagocytic capacity (Gordon and Katsh, 1949; Holden, Seegal and Ryby, 1951; Nicol and Snell, 1954).

When solid tumours or tumour mince is employed as transplantation material, a proliferation of the malignant cells will require a supply of stroma from the host. Generally, this appears to be one of the worst obstacles to successful transplantation of heterologous tumours. Clemmesen in his monograph (1938) ascribes the growth of mouse tumours obtained in his experiments on roentgen irradiated rats largely to the use of tumours primarily poor in stroma. Furthermore, as cortisone is known to have a restrictive influence on the formation of connective tissue (Ragan, Howes, Plotz, Meyer and Blunt, 1949; Spain, Molomut and Haber, 1950; Cornman, 1951; Cavallero, Borasi, Sala and Amira, 1951; Taubenhaus, 1953), the possibilities of stroma formation, and thus of growth of the graft, will be very poor in animals treated with the hormone. Correspondingly in the author's experiments no proliferation of tumour cells was seen in solid grafts.

However, in one of the two experimental series on human ascites with tumour cells needing no stroma, an ascites tumour developed in mice treated with cortisone. On serial transmission these tumour cells seemed to have adapted themselves to such an extent that on and after the third passage no cortisone treatment was necessary, as the procreative power of the cells had increased through the passages. It was only after this time that the cells proved capable of forming solid masses and thus of obtaining a scanty stroma support from their new host, whether treated with cortisone or not (Fig. 8).

It appears from studies by Klein (1951) that intra-abdominal implantation may facilitate the taking of certain tumours, and the positive result from intraperitoneal grafting of ascitic fluid here reported might not be accidental.

However, the morphological conformity between ascites tumour from mice and humans may perhaps be a coincidence. But that the ascites tumour in the mice should be spontaneous and independent of the transmitted human cells would seem unlikely, as cells of identical type developed in 4 out of 9 cortisone-treated mice, and the first serial transmissions required animals with lowered capacity of antibody formation. Besides, spontaneous ascites tumours in this mouse strain have never been observed in the present laboratory.

To the author it seems more likely that a survival in the human ascites of cells with marked capacity of mutation has resulted in a selection of cells particularly suited for this kind of transmission, but the chromosome types in the ascites tumour is now under further analysis.

SUMMARY

Attempts at transplantation of 21 different human neoplasms to cortisone-treated mice are recorded. After injection of tumours minced with scissors, tumour tissue survived for 10–45 days in treated animals in 11 out of 19 series. Though apparently capable of living, the malignant cells did not show proliferation or invasiveness, probably chiefly because of difficulties in obtaining stroma from the new host.

In one of two series where human ascites, containing tumour cells, was used as transplantation material, an ascites tumour formed in 4 out of 9 cortisone-treated mice. This tumour, till now (March 1956) carried in 41 passages, has shown increasing growth rate, and from the third passage it was able to grow also in untreated animals, and to form infiltrating solid tumour masses in the latter. Thus, human tumour cells seem to have been established in normal experimental animals.

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EXPLANATION OF PLATES

FIG. 1.—Metastatic skin tumour from human adenocarcinoma of oesophagus (Patient No. Z.1519). Haematoxylin-eosin. × 250.

FIG. 2.—Transplantation site in a mouse 45 days after subcutaneous grafting of human oesophageal adenocarcinoma shown in Fig. 1. Note preservation of structure. Haematoxylin-eosin. × 250.

FIG. 3.—Breast cancer of the patient No. R.64561. Adenocarcinoma with marked mucoid degeneration. Haematoxylin-eosin. × 65.

FIG. 4.—Solid carcinoma of cervix from patient No. R.64561. Haematoxylin-eosin. × 250.

FIG. 5.—Ascites smear from patient No. R.64561. Large number of tumour cells varying in size and shape. Pronounced admixture of granulocytes. Haematoxylin-eosin. × 365.

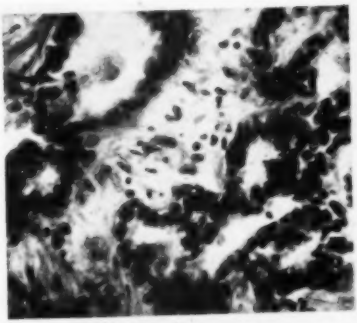
FIG. 6.—Three cortisone-treated mice and one control (left) 45 days after intra-abdominal inoculation of human ascites (from patient No. R.64561). Marked abdominal distention in all the treated mice.

FIG. 7.—Ascites smear from a cortisone-treated mouse 35 days after inoculation of the patient's ascites fluid. Tumour cells varying in size, shape and in number of nuclei. Pronounced vacuolation of the cytoplasm. Haematoxylin-eosin. × 425.

FIG. 8.—Solid tumour growth in a cortisone-treated mouse from the third passage of the ascites tumour H.A.1. Note existence of capillaries and the scarcity of stroma. Haematoxylin-eosin. × 300.

FIG. 9.—Tumour infiltration of kidney in an untreated mouse from the third passage of the ascites tumour H.A.1. Haematoxylin-eosin. × 300.

FIG. 10.—Tumour infiltration of pancreas in an untreated mouse from the fourth passage of the ascites tumour H.A.1. Haematoxylin-eosin. × 300.



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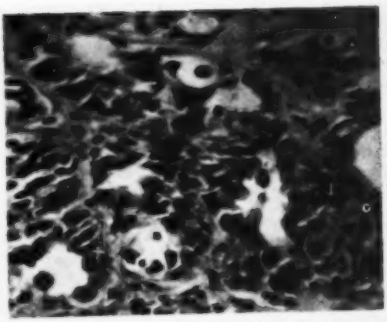

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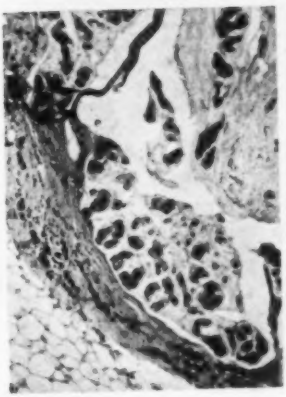

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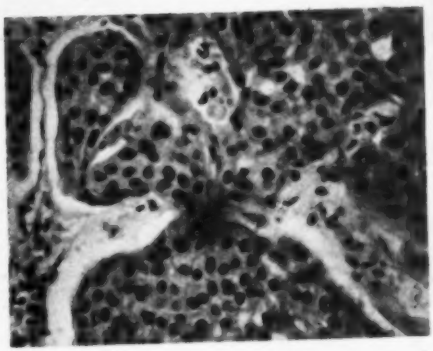

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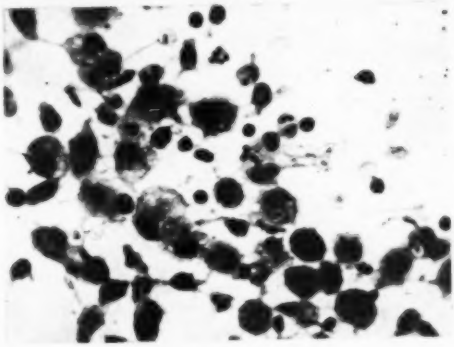

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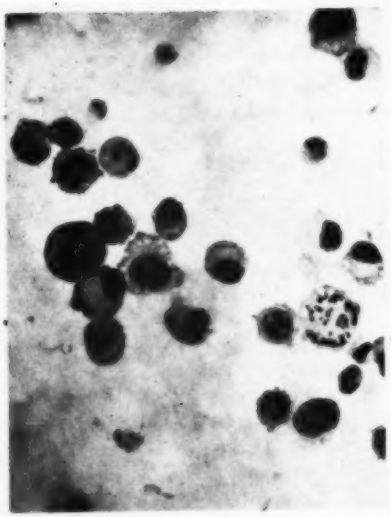

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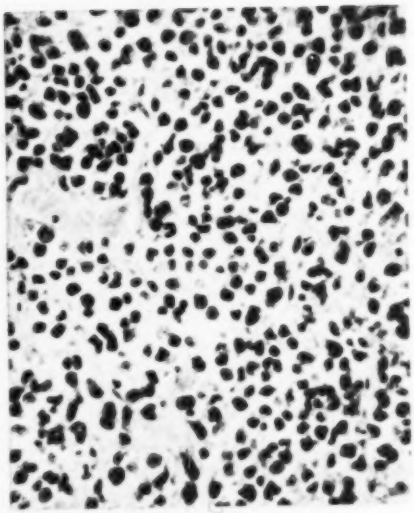

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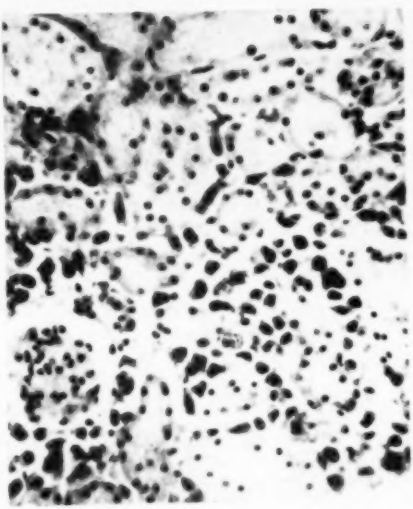

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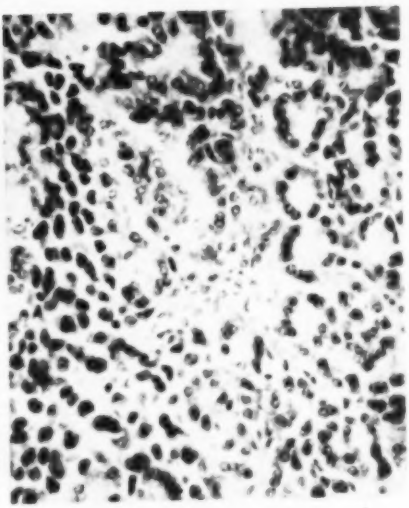

10

THE DEGREE OF AIR POLLUTION IN NORWEGIAN TOWNS

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AN increasing effect of a general air pollution is repeatedly advanced as a possible explanation of the increasing incidence of lung cancer observed in this century. An important argument in this connection is the universal finding of a difference in lung cancer frequency in larger towns, smaller towns and rural districts.

Among the substances especially suspected as active in this connection, 3 : 4-benzpyrene occupies a prominent position, and in a recent paper Stocks and Campbell (1955) give figures from England and Wales indicating amounts of benzpyrene assumed to be inspired from cigarette smoke and from air inhaled during ordinary breathing.

In this connection it may be of interest to compare the incidence of lung cancer and the degree of air pollution, the latter expressed by the content of benzpyrene, in a country showing marked differences from England and Wales.

Upon the suggestion of Sir Ernest Kennaway, a co-operation was initiated some years ago, to find the degree of air pollution at a few sampling posts in Norway, using the same technique as used in the pioneer work in England.

The main sampling post was the laboratory premises in the University hospital (Rikshospitalet, Oslo) situated in the centre of the town. The site of Rikshospitalet, as well as the smoke-producing industrial plants, have been indicated in Fig. 1. The neighbourhood of the hospital is a residential area with a rather dense population, living in flats built some 70–80 years ago, where heating is effected partly by electricity, but mainly by coke and oil (central heating).

The air at the site of the hospital should represent a very fair average of the daily air breathed by the inhabitants of the denser Oslo.

The air was, by means of an ordinary water suction apparatus, drawn through a Whatman Filter Paper No. 50, and the amount of air measured on an ordinary gas meter. For each day the meteorological conditions were noted. The papers were examined by Campbell, using the technique described in the paper by Stocks and Campbell (1955).

The findings for Oslo are given in Table I and in Fig. 2 and 3.

It will be seen how the amount of smoke (suspended impurities) with its content of polycyclic hydrocarbons varies during a twelve-month period. Benzpyrene occurs in considerably greater relative amounts during the autumn-winter months as compared to the amounts during the spring-summer months.

The figures for Oslo are of the same order of magnitude as the figures from Copenhagen (Campbell and Clemmesen, 1956), and the situation for Oslo may, when compared to the situation in England and Wales (Campbell and Stocks, 1955) be summarized as follows ;

TABLE I.

	Oslo.			
	Feb.–March (1955).	Apr.–Sept. (1955).	Oct.–Nov. (1955).	Dec. (1955) to Jan. (1956).
	Parts/million.			
Anthracene	8	4	9	5
Pyrene	51	108	104	155
Fluoranthene	52	120	69	119
3 : 4 Benzpyrene	152	155	295	292
1 : 12 Benzperylene	143	220	176	199
	μg./100 m.³.			
Anthracene	0·021	0·001	0·025	0·025
Pyrene	0·121	0·056	0·430	0·790
Fluoranthene	0·116	0·079	0·290	0·620
3 : 4 Benzpyrene	0·345	0·086	1·240	1·520
1 : 12 Benzperylene	0·358	0·122	0·690	1·010
Smoke mg./m.³	0·020	0·006	0·045	0·051

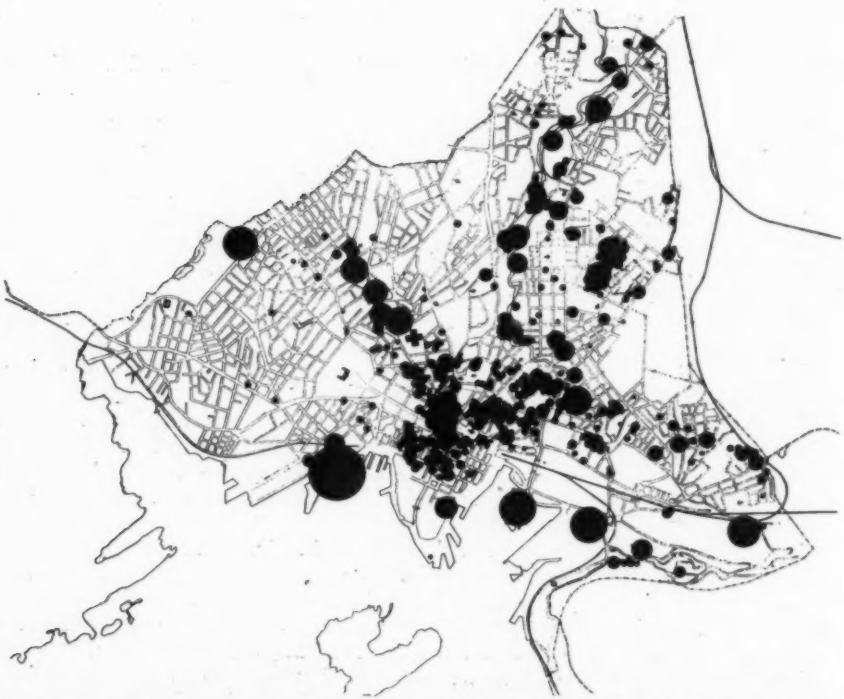

FIG. 1.—City plan of Oslo. The dark markings indicate the size of the industrial plants, based upon the number of workers employed. The cross marks the site of Rikshospitalet, the sampling post.

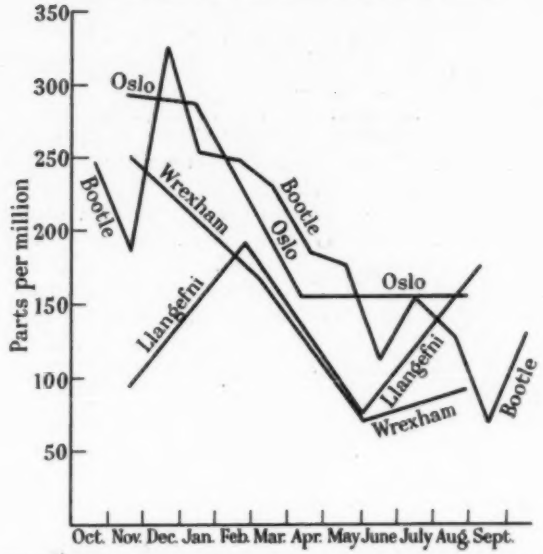

Fig. 2.—Yearly concentration of 3 : 4 benzpyrene in suspended impurity in Oslo in 1955 compared with Bootle, Wrexham and Llangefni—in England and Wales.

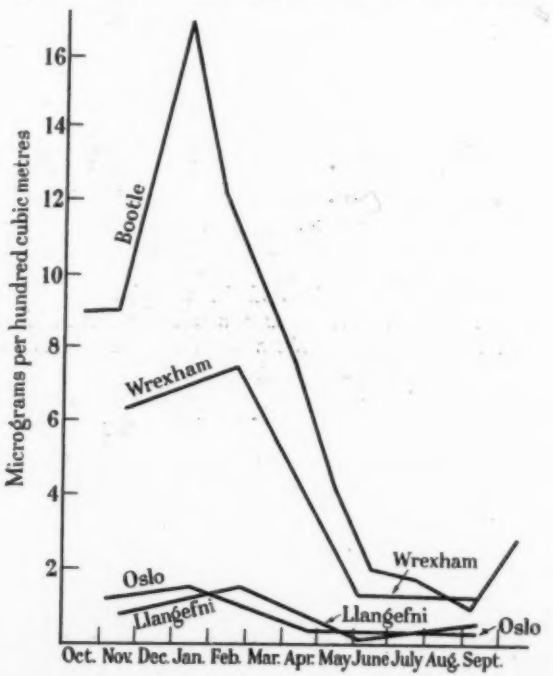

Fig. 3.—Yearly concentration of 3 : 4 benzpyrene in the air at Oslo in 1955 compared with Bootle, Wrexham and Llangefni in England and Wales.

(1) The relative amounts of benzpyrene in the suspended matter filtered from the air of Oslo is of the same order of magnitude as that found in samples from representative towns in England and Wales.

(2) The absolute amount of benzpyrene present in the Oslo air is of the same order of magnitude as that of a coastal Welsh village (Llangefni).

(3) The concentration of benzpyrene follows similar seasonal trends with a maximum in winter and a minimum in summer.

Scattered observations were made in Bergen, Halden (a commercial, non-industrial small town) and Notodden (a small industrial town) (Table II). The observations from Bergen show conditions similar to those of Oslo, but the absolute amounts of benzpyrene are lower. The figures from Halden and Notodden show still lower figures, lower than any observed in any locality in England and Wales.

TABLE II.

	Bergen. 1 (Jan., 1955).	Bergen. 2 (Mar., 1955).	Halden. Dec. (1954).	Notodden. Jan. (1955).
	Parts/million.			
Anthracene	50	—	—	—
Pyrene	61	48	131	145
Fluoranthene	103	78	75	186
3 : 4 Benzpyrene	275	122	180	317
1 : 12 Benzperylene	133	193	97	85
	μg./100 m.^{3}.			
Anthracene	0·36	—	—	—
Pyrene	0·43	0·185	0·40	0·115
Fluoranthene	0·72	0·29	0·23	0·147
3 : 4 Benzpyrene	1·91	0·465	0·56	0·250
1 : 12 Benzperylene	0·92	0·74	0·30	0·087
Smoke mg./m.^{3}	0·082	0·037	0·003	0·008

Bergen sampling post 1 : Den Tekniske Skole. 2 : Fiskerilaboratoriet.

SUMMARY

3 : 4 Benzpyrene has been estimated in samples of atmospheric smoke (suspended matter) in Oslo and a number of smaller towns in Norway, and these results are compared with similar values obtained for towns in England and Wales.

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3,4–BENZPYRENE IN THE SMOKE OF CIGARETTE PAPER, TOBACCO, AND CIGARETTES*

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RECENT statistical studies suggest a relationship between the increasing incidence of lung cancer and smoking. This implies carcinogenic activity by the smoke (Doll and Hill, 1950 ; Hammond and Horn, 1954 ; Wynder and Graham, 1950). Accordingly, for several years this laboratory has been investigating the nonvolatile fractions of the smokes of cigarettes, cigarette paper, and tobacco for possible known carcinogens. The statistical studies (Hammond and Horn, 1954) on the relation of lung cancer and smoking pointed especially at cigarette smoking and indicated little or no relation to cigar and pipe smoking. One major difference between these types of smoking is, of course, the cigarette paper. Initially, it was thought the carcinogenic activity might be wholly due to the paper, and we were thus stimulated to start with the paper alone.

Indications of fluorescence, characteristic of the benzanthracene derivatives, was first noted by Carroll and Rand (unpublished observations) in the tars from cigarette paper smoke. The fluorescence was found by us to be due to 3,4-benzpyrene.

The technique for burning the paper was not designed to simulate the conditions of actual cigarette smoking. The paper was smouldered in a stream of air (Fig. 2), the highest temperature reached at the burning front (650–950° C.) being in the same range as that attained in a cigarette during inhalation (Wynder, Graham and Croninger, 1953). It was felt that the combusion products would vary with temperature of burning but that qualitatively the same products would be formed. Work subsequently published by Cooper and Lindsey (Cooper and Lindsey, 1954 ; Cooper, Lindsey and Waller, 1954) on cigarettes made wholly of cigarette paper without tobacco and smoked in an apparatus designed to simulate actual smoking conditions supports this assumption ; 3,4-benzpyrene was obtained by these workers although in smaller yields than obtained by us. Additional substantiation was provided by the work on the whole cigarette ; the quantity of 3,4-benzpyrene produced was in line with that expected from the results on the paper and tobacco burned separately.

Subsequently we found 3,4 benzpyrene in the smokes of tobacco, cigarettes, and cigars. The cigarettes and cigars were smoked in a smoke sampling apparatus designed to approximate average conditions of actual smoking.

Much of the work reported here is a duplication with some extension of similar work by Lindsey and Cooper and is further confirmation of their results.

* Material from this paper has been presented at Regional Meeting of South-Eastern Section of Amer. Chem. Soc., Birmingham, Ala., Nov., 1954; Tobacco Chemists Meeting, Raleigh, N. Car. Oct., 1955 ; and Annual Meeting of AAAS, Atlanta, Ga., Dec., 1955.

EXPERIMENTAL

Apparatus and Chemicals

Benzene.—Thiophene free.

Cyclohexane.—Practical. This solvent was purified by freezing 4/5 of it and discarding the supernatant liquid.

Alumina.—Harshaw chromatographic grade, heated to 130° C. in an oven for several hours before use. For the iodination experiment, Alcoa F-20 alumina, heated to 130° C. for 1 hour, was used.

Tobacco.—Popular brand of pipe and cigarette tobacco.

Cigarettes.—Popular brands.

Cigars.—2 small size and 2 regular size ; popular brand.

Silica gel.—28–200 mesh. Fisher Scientific.

Glass wool.—Pittsburgh Plate Glass Co. SE 3 × 5—621 glass unbonded B.

Cigarette paper.—Commercial brand.

3,4 benzpyrene.—Eastman.

Methylcholanthrene.—Eastman.

Dibenz(ah)anthracene.—Eastman.

1,12 benzpyerylene.—Aldrich Chemical Co.

Initially, a Beckmann DU ultraviolet spectrophotometer was used, then a Beckmann DK-1 recording spectrophotometer for fluorescence and ultraviolet absorption work. For fluorescence (Burdett and Jones, 1947) the light housing backplate was modified to make the fluorescence sample the light source (Fig. 1). A small platform with a holder for a vial 1 cm. in diameter extended from the backplate, placing the vial at the same position normally occupied by the filament of the tungsten lamp. The ultraviolet exciting light was provided by a GE mercury vapor lamp (CH3) with appropriate transformer. This light passed through a hole in the backplate covered by a glass light filter (Corning 5860) which permitted light under 390mμ to enter the lamp housing. The mercury vapor lamp and transformer were mounted in a metal box provided with a hole adjacent to the lamp. A strip of aluminium foil was fitted to the back half of the lamp to serve as a crude reflector. The " energy " setting of the spectrophotometer and high sensitivity were used for fluorescence measurements.

Smoke Sampling

Cigarettes and cigars were smoked in a smoke sampling apparatus designed by the research laboratory of The American Tobacco Company, Inc. and manufactured by Phipps and Bird, Inc. of Richmond, Virginia. In this apparatus each cigarette was smoked individually, one 3-second inhalation of 60 c.c. of air each minute. The smoke was drawn through acetone. Four cigarettes were smoked simulataneously.

Cigarette paper and tobacco were smouldered in a glass tube 1¼ inches in diameter and 12 inches long (Fig. 2). This tube was connected by means of a

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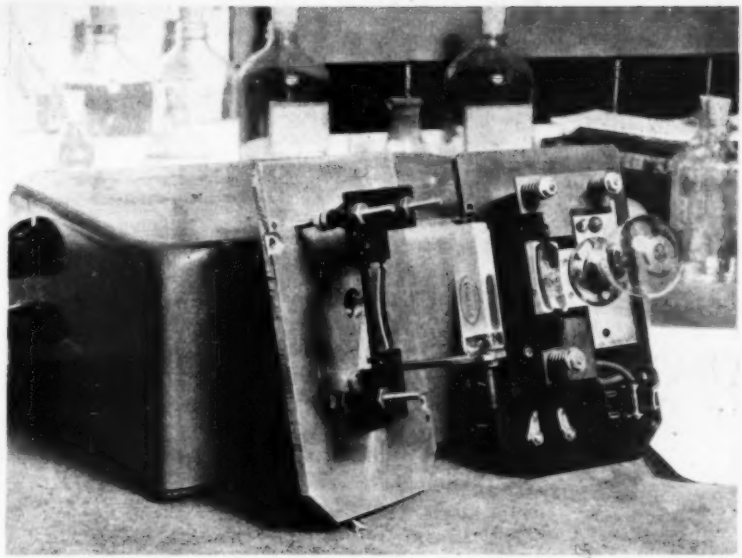
EXPLANATION OF PLATE.

FIG. 1.—Beckmann spectrophotometer modified to make the fluorescence sample the light source.

FIG. 2.—Vertical glass tube 1¼ inches diameter and 12 inches long in which cigarette paper and tobacco were smouldered.



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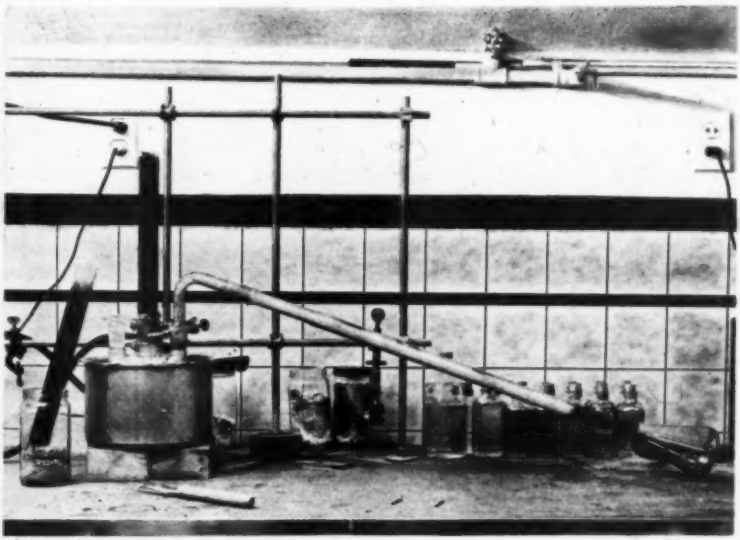

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34/45 joint to the center neck of a 3-neck flask set in a water bath at room temperature. A piece of wire gauze was placed toward the bottom of the burning tube to prevent the ashes from falling into the flask. To the flask was fitted an adaptor leading to a glass tube tapered at the lower end containing a wad of glass wool 1 to 2 inches long. The tapered end of the condenser tube passed through a rubber stopper into a 200 c.c. filter flask. The filter flask was connected by vacuum tubing to a trap and then to a water aspirator.

Chromatography

Glass tubes 1½ inches to 1 cm. in diameter and 2 to 3 feet long were used. Eluents were collected in narrow-mouth glass bottles 500 to 15 c.c. capacity.

Procedure

1. Cigarette Paper

Cigarette paper was unwound from a commercial roll in 5 to 6 feet lengths. Thirty such lengths (about 30 g.) were wound together and torn into approximately 12 inch lengths. The burning tube was loaded with these wads. With the aspirator on full, the paper was lit and allowed to smoulder down to ashes. The burning front was periodically measured with a bare thermocouple junction and the temperature found to vary between 650 and 900° C. Temperatures to 850° C. were measured with an optical pyrometer. The air flow measured with a flowmeter averaged 5 l. per minute. Thirty minutes was the average time required for the complete burning of the 30 g. samples of paper and about 150 l. of air was passed. This corresponds to 30 l. of oxygen or 1·3 mols. or 7 mols. 0_2/162 g. of cellulose. Theoretically, 6 mols of oxygen are required for the complete combustion of each 162 g. unit of cellulose so that an excess of oxygen was always used in these experiments.

Ten batches (300 g.) of paper were burned in an average day's experiment. Reddish tarry condensate accumulated on the sides of the flask, connecting tube, condenser, and especially in the glass wool plug. Liquid condensed in the glass wool and dropped into the filter flask. When 300 g. of paper had been burned, the apparatus was dismantled and rinsed down with 300 to 500 ml. of acetone (except for the burning tubes), which dissolved all of the condensed tars. The nonvolatile tars produced by the smouldering paper were determined by evaporating a sample of the acetone solution to dryness on a steam bath. Fifteen to 18 g. (5 to 6 per cent) of tars were obtained.

To the acetone solution in a separatory funnel, 500 ml. of cyclohexane was added, followed by 500 ml. of water. The aqueous-acetone water layer was drawn off and discarded. The cyclohexane solution was washed with two 500 ml. portions of water and dried over amhydrous calcium chloride. The dried cyclohexane solution was a sharp smelling, irritating reddish-brown liquid.

A 1 × 6 inch column of Harshaw chromatographic alumina, activated by heating in an oven at 130° C. for several hours, was prepared. The cyclohexane solution was added to the column and it was followed by pure benzene. The cyclohexane passed through the alumina, leaving all the colored material in a narrow band within 1 inch of the top of the column. If the alumina was insufficiently activated, it could be noted by the fact that the colored material spread through the column and came through in the cyclohexane eluents.

The addition of benzene started a yellow material down the column. When irradiated with ultraviolet light, this yellow material fluoresced a bright blue. Fifty milliliter eluents were collected and the fluorescence spectra of all fractions determined. The first benzene eluents were yellowish-green and fluoresced in one wide bright band with a peak at 440–450 mμ (Fig. 3). At the fourth or fifth benzene eluent, two peaks could be discerned at 410 to 412 and 432 to 435 (Fig. 4). The fluorescence spectrum of pure 3,4-benzpyrene in benzene solution has three major peaks at 410, 432, and 455 mμ, with a smaller peak at 413 on the shoulder

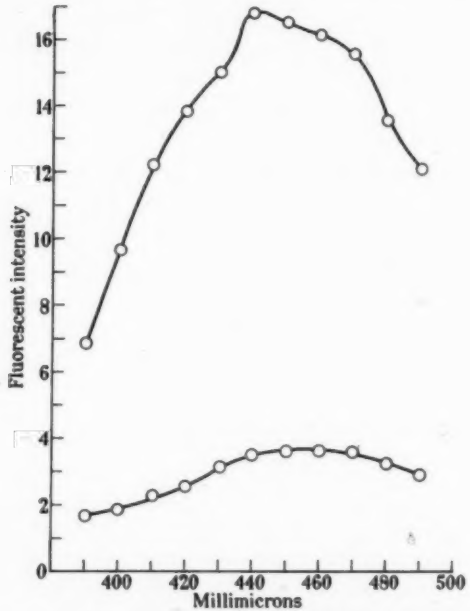

FIG. 3.—Fluorescent spectra of eluents from the cigarette paper tar separation which do not contain 3,4-benzpyrene.

of the 410 peak. The 2 peak fluorescence noted in the benzene eluents of the chromatographic column was the first indication of the presence of 3,4-benzpyrene or other benzanthracene derivatives in the tar. Five to 6 eluents (about 300 ml.) had indications of the 2 peak fluorescence. Subsequent eluents fluoresced with a wide band background fluorescence. Much dark brown material remained on the column.

The eluents showing the 2 peak fluorescence were combined and evaporated to dryness in vacuo at 50 to 60° C. and the residue dissolved in 50 ml. of cyclohexane. This solution was added to a column, $\frac{3}{4} \times 4$ inches, of silica gel. A colored band remained within $\frac{1}{2}$ inch of the top of the absorbent layer. The column was developed with a solution of 10 per cent benzene in cyclohexane. Twenty-milliliter eluents were taken. The first benzene containing eluents fluoresced in bands with two prominent peaks at 410 and 432 mμ and a flat spot

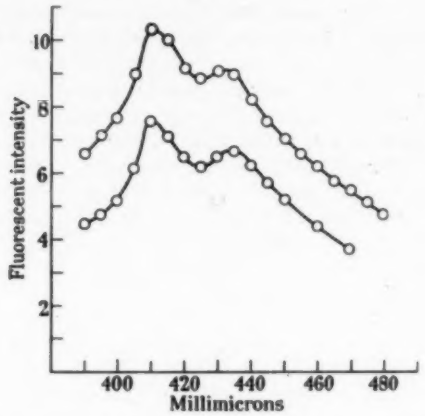

FIG. 4.—Fluorescent spectra of eluents from the cigarette paper tar separation which contains 3,4-benzpyrene.

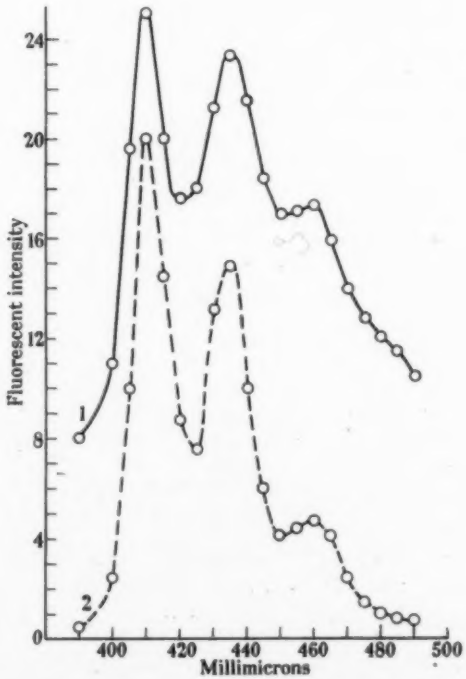

FIG. 5.—Fluorescent spectra. 1. Purified fraction of cigarette paper tar rich in 3,4-benzpyrene. 2. 3,4-Benzpyrene.

at 450 to 455 mμ. The ultraviolet absorption curve showed a small peak at 385 and flat spots at 360 to 370 and 345 to 350 mμ. Three hundred to 400 ml. were obtained.

The eluents with the 2 peak fluorescence were combined, evaporated to dryness and the residue dissolved in 25 ml. of cyclohexane. The cyclohexane solution was added to an alumina column 1 cm. in diameter and 2 inches long. The column was developed with 10 per cent benzene in cyclohexane. Ten milliliter eluents were taken. The ultraviolet absorption curves in eluents (15 to 25) showed definite peaks at 385 and 365 and suggestions of peaks at 347 and 405 mμ.

The eluents with the suggestive ultraviolet absorption were combined, evaporated to dryness and taken up in 25 ml. of cyclohexane. This solution was added to a column of silica gel 1 cm. by 3 inches long. The column was developed with 5 per cent benzene in cyclohexane. The first benzene containing eluents had sharply defined peaks in the ultraviolet absorption spectra at 365, 385, and 405 with a flat spot at 345 to 350 mμ. There was a flat shoulder at 380 mμ just below the peak at 385 mμ. Two additional small columns of silica gel, developed with 3 and 1 per cent benzene in cyclohexane solutions, gave eluents with more clearly defined 3,4-benzpyrene absorption. Peaks at 333 and 317 suggest 1,2-benzpyrene is also present in these samples (Fig. 12). The fluorescence spectrum was very close to that of 3,4-benzpyrene (Fig. 5 and Fig. 6).

Quantitative Estimation of the 3,4 *Benzpyrene*

The eluents from the last chromatographic column were combined and an ultraviolet absorption curve run on the resulting solution (Fig. 12). An estimate was made of the 3,4-benzpyrene content of the solution using the method described in detail by Cooper (1954). In this method, linear background absorbance is asssumed over a small range under the most prominent peak of 3,4-benzpyrene (385 mμ). A base line is drawn from a point (A) on the absorption curve at 375 mμ to another point (B) on the curve at 395 mμ. A vertical line is dropped from the 385 peak (C) to its intersection (D) with the line. The absorbance at (D) is subtracted from the absorbance of the curve at 385 mμ (C). This is done for a solution of known concentration of 3,4-benzpyrene and for the solution whose concentration is to be determined ; then,

Known absorbance/Unknown absorbance
= Concentration known/Concentration unknown.

In the sample shown, an eluent solution of 300 ml. from the chromatography of tars from 550 g. of cigarette paper gave the curve in Fig. 12. A solution of 0·001 per cent 3,4-benzpyrene in cyclohexane gave the other curve in Fig. 12.

	0·001% 3,4-benzpyrene in cyclohexane.		Eluent solution.	
	C.	D.	C.	D.
% transmission	10·8	55·2	43·0	62·1
Absorbance	0·967	0·258	0·367	0·207
$A_C - A_D$	0·709		0·160	

The percentage of 3,4-benzpyrene in the unknown solution was determined by substituting in the equation

$$\frac{0{\cdot}160}{0{\cdot}709} \times 10\ \mu g. \times 150 = 337{\cdot}2\ \mu g.$$

$$\frac{550}{0{\cdot}000337} = 1 \text{ part benzpyrene per } 1{,}610{,}000 \text{ part of paper burned.}$$

To check the estimation method, 150 g. of paper were smouldered as above. The apparatus was washed with acetone. The acetone solution was divided in two equal portions. To one portion was added 5 ml. of a solution containing 10γ

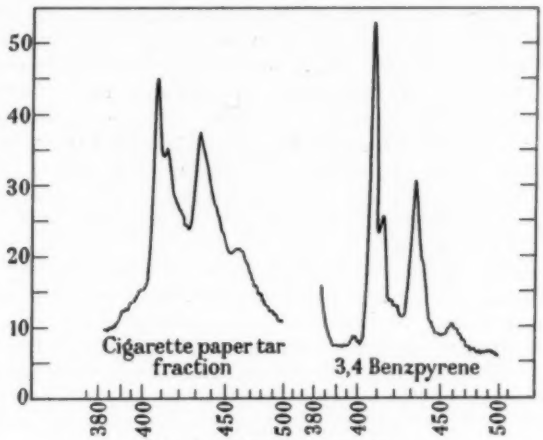

FIG. 6.—Fluorescent spectra.

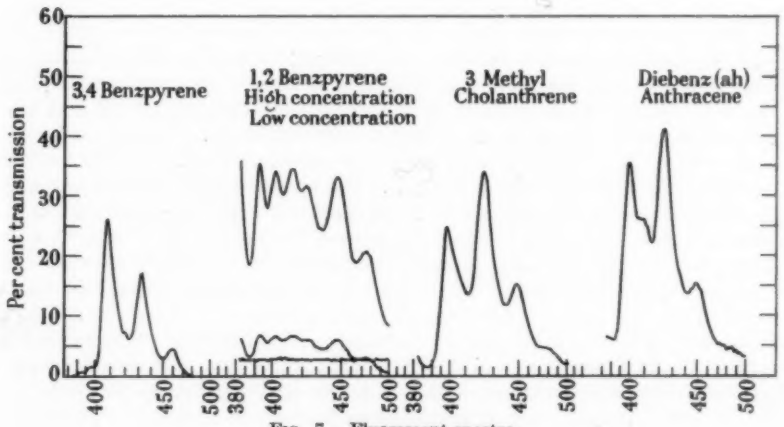

FIG. 7.—Fluorescent spectra.

of 3,4-benzpyrene per milliliter of cyclohexane or a total of 50 γ of 3,4-benzpyrene. The two portions of the acetone solution were chromatographically analyzed using the procedure outlined above. Ninty-two γ of 3,4-benzpyrene were obtained from one portion and 44 γ from the other. Thus, 48 γ of the added 50 were recovered.

6-Iodo 3,4-*Benzpyrene**

* See Tye, Graf and Horton (1955).

A solution obtained by chromatography of tars from cigarette paper smoke containing 97 γ of 3,4-benzpyrene was evaporated to dryness. To a solution of the residue in 10 ml. of benzene was added half of a solution prepared by dissolving 1 g. of iodine in 10 ml. of benzene. The other half of the iodine solution was added to a $\frac{3}{4} \times 1\frac{1}{2}$ inch column of activated Alcoa F-20 alumina. The benzene solution of iodine and cigarette paper tar was then poured on to the column followed by pure benzene. The first benzene eluents containing iodine were shaken in a separatory funnel with a 5 per cent solution of sodium thiosulfate containing a crystal of potassium iodide. The decolorized benzene solution gave peaks in the ultraviolet absorption spectra at 402, 382, and 367 mμ. The 402 peak was now the most prominent, whereas in the pre-iodination sample it was only a very small peak. The absorption of subsequent samples reverted to the 3,4 benzpyrene absorption; evidently 6-iodo 3,4-benzpyrene is held more loosely by the alumina and thus comes off before 3,4-benzpyrene.

In order to enrich the 6-iodo 3,4-benzpyrene in the eluents, they were combined, evaporated to small volume and rechromatographed over a $\frac{1}{2} \times 3$ inch column of Alocoa F-20 alumina. The column was eluted with 10 per cent benzene in cyclohexane. A further separation of the iodo compound from unreacted 3,4 benzpyrene was effected. The first eluent contained an unidentified material with peak absorption at 330 mμ, and was followed by eluents rich in the iodo compound, although still containing unidentified material with background absorption and some 3,4-benzpyrene (Fig. 8).

II. Tobacco

Tobacco (from a popular brand of cigarettes), 120 g. in 30 g. batches, was smouldered in the same apparatus as used above for the cigarette paper (Fig. 2). After each 200 g. had been smouldered, the apparatus was dismantled and washed down with acetone. The acetone solutions were combined. The acetone solution (one liter) was diluted with one liter of cyclohexane followed by one liter of water. The aqueous-acetone layer was discarded. The cyclohexane solution was washed twice with water, with 600 ml. of 2 N HCl, and then with water until neutral.

The dried cyclohexane solution was passed through a $1\frac{1}{2} \times 10$ inch column of silica gel. The cyclohexane eluents were dark in color but were discarded after further chromatography failed to disclose the presence of any 3,4 benzpyrene. The origianl cyclohexane solution was followed on the column by 100 ml. of fresh cyclohexane and then by 800 ml. of benzene. The benzene solution fluoresced with high over-all fluorescence in the 400 to 450 mμ range, but indications of 3,4 benzpyrene fluorescence were not obtained because of masking by the background.

The benzene solution was evaporated to dryness in vacuo at 50–60° C. and the residue dissolved in 100 ml. of cyclohexane. This solution was added to a $\frac{3}{4} \times 4$ inch column of alumina and followed by pure benzene; 20 ml. eluents were collected. Two hundred milliliters were obtained with 2 peak fluorescence at 410 to 420 and 435 to 445 mμ superimposed on a high background fluorescence.

These eluents were combined and evaporated to dryness as above. The residue was taken up in 50 ml. of cyclohexane and this solution added to a $\frac{3}{4} \times 5$ inch column of silica gel. The column was eluted by a solution of 10 per cent benzene in cyclohexane. The 2 peak fluorescence was improved but there were only slight breaks in the absorption curves at 385 mμ.

The eluents showing 2 peak fluorescence were again combined, evaporated to dryness and the residue dissolved in 20 ml. of cyclohexane. This solution was chromatographed on alumina, 1 × 8 cm., eluting with 10 per cent benzene in cyclohexane. The first 200 ml. of eluents showed nothing. The next 50 to 100 ml. gave an absorption peak at 375 mμ probably due to anthracene. These were followed by eluents with peaks at 405, 385, and 365 mμ. Later eluents showed a peak at 383 mμ, possibly a derivative of 3,4-benzpyrene.

The eluents with the suggestive absorption were combined, evaporated to dryness in vacuo and the residue taken up in 25 ml. of cyclohexane. The next column was a small silica gel one, 1 × 8 cm.; the eluting solution was 10 per cent benzene in cyclohexane (10 ml. eluents). The absorption curves improved in eluents 5 through 12. A second silica gel column as above gave further improved absorption curves. The curve shown (Fig. 9) is the absorption of the residue from the appropriate eluents dissolved in 65 ml. of cyclohexane. Using this curve and the analytical method described above for cigarette paper, 152 μg. of 3,4-benzpyrene were obtained.

$$\frac{\log \frac{1}{0{\cdot}30} - \log \frac{1}{0{\cdot}44}}{\log \frac{1}{0{\cdot}108} - \log \frac{1}{0{\cdot}565}} \times 65 \text{ ml.} \times \frac{10\ \gamma}{\text{ml.}} = 152\gamma$$

$$\frac{1250}{0{\cdot}000152} = 1 \text{ part benzpyrene per } 8{,}200{,}000 \text{ parts of tobacco burned.}$$

III. Cigarettes

Cigarettes (three popular brands) were separately smoked in the cigarette smoke-sampling apparatus. Four hundred cigarettes were smoked in an experiment. The separation and identification technique for the tars was the same as that used on the tobacco tar. Estimates of 3,4-benzpyrene content were as follows : Brand A—40 μg.; Brand B—49 μg.; Brand C (filter tip, king size)—32 μg.

The estimation method was checked as in the case of the cigarette paper by adding 10 μg of 3,4-benzpyrene to one half of the acetone solution of tars from 380 cigarettes. Fifteen micrograms of 3,4-benzpyrene were obtained from one portion and 23·5 μg from the other ; the recovery was thus over 85 per cent of that added.

Indications of iodo-benzpyrene were obtained by conducting the catalytic iodination experiment on a purified fraction of the cigarette tars as described with the cigarette paper above.

IV. Cigars

The smoking apparatus was that used for cigarettes, although modifications in the size of the tubes used as cigarette holders were necessary to accommodate the larger size cigars. Regular size and small size cigars were separately smoked. The separation and identification was that used on the tobacco described in the foregoing. The results were :

Brand A (regular size)	82·5 γ per 20 cigars (wt. of cigar 8 g.)
Brand A (small size)	94·0 γ per 49 cigars (wt. of cigar 2·75 g.)
Brand B (small size)	10 γ per 24 cigars (wt. of cigar 2·75 g.)
Brand C (regular size)	25 γ per 24 cigars (wt. of cigar 8·5 g.)

In the eluents from Brand A containing 3,4-benzpyrene, a prominent peak is present at 340 mμ. This may be due to coronene.

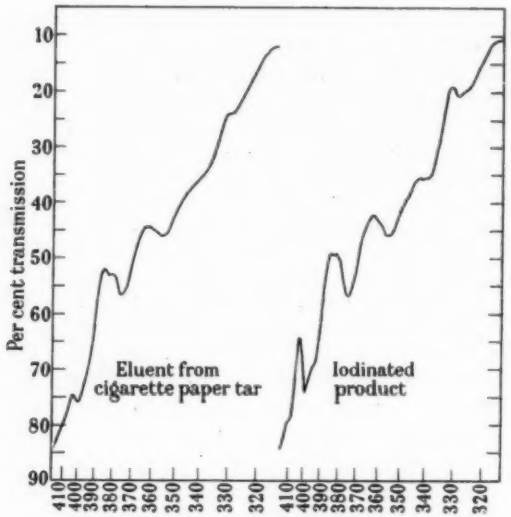

FIG. 8.—UV absorption.

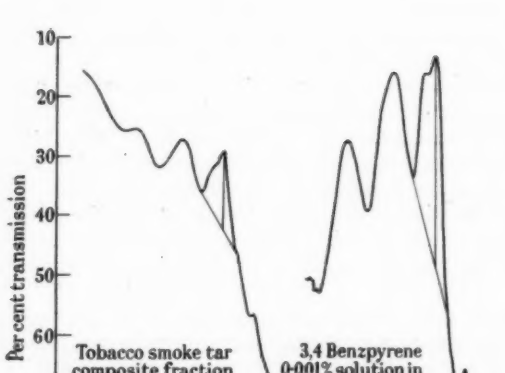

FIG. 9.—UV absorption.

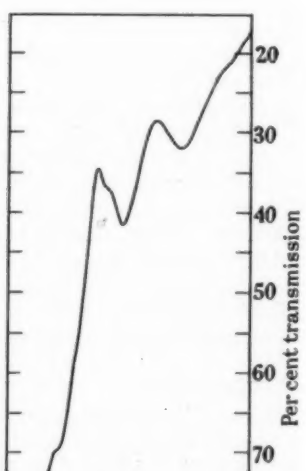

FIG. 10.—UV absorption; cigarette tars composite fraction.

DISCUSSION

The identification of 3,4 benzpyrene in the tars from the smoke of cigarettes, tobacco, and cigarette paper is based on four considerations.

1. The fluorescence spectrum. The original crude spectra of the first investigators in this field could hardly be considered identification, since they reported 3 bands in the wavelength region, 4000 to 4500 Å (Cook, Hewett and Hieger, 1933; Kennaway and Hieger, 1930). The spectra recorded with a wide slit show only 3 bands, but even so a difference is readily apparent between 3,4-benzpyrene, 1,2,5,6 dibenzanthracene, and 10 methylcholanthrene (Fig. 7). The narrow slit fluorescence spectrum discloses a still more complex spectrum for 3,4-benzpyrene (Fig. 6); 4 distinct peaks at 406, 408, 430, and 450 with fine irregularities in the region 410 to 420 mμ. This fine structure fluorescence spectrum of 3,4-benzpyrene was first noted by Bernard Muel and Michel Hubert-Habart at The Institute du Radium in Paris, France. The apparatus for fluorescent spectra used in that laboratory was designed by the director of the laboratory, R. Latarjet, and is capable of high sensitivity and high dispersion. Nevertheless, much more work is necessary before fluorescence itself can be considered final identification, since carefully determined spectra of many 3,4-benzpyrene-like compounds must yet be done.

2. Ultraviolet absorption spectrum. The identification using absorption spectra is more certain than is fluorescence alone. Five distinct peaks can be seen, at 405, 385, 380, 365, 347, with a discontinuity at 395 mμ. The spectra of only two rare polynuclear hydrocarbons of many that have been reported are closely enough aligned to that of 3,4 benzpyrene to raise serious doubts of the identity (9-methylanthracene and 1 : 12-benzperylene) (Friedel and Orchin, 1951). Fluorescence eliminates the latter as its fluorescence spectrum is completely different from that of 3,4-benzpyrene (Fig. 11).*

3. The indication of the presence of 6-iodo 3,4-benzpyrene in iodinated purified fractions of the tars (Tye, Graf and Horton, 1955).

4. The recovery of added quantities of pure 3,4-benzpyrene in the same eluents by the identical procedures as with the substance being investigated.

Further, there is indication of the presence of 1,2-benzpyrene (absorption peaks at 333 and 317 mμ) in the same eluents with the 3,4-benzpyrene, as would be expected. This is especially clear and pronounced in the excellent absorption curve obtained by Lefemine at the Miami Cancer Institute, on a highly purified fraction of cigarette paper tar.

The weight of a cigarette is about 1 g. The weight of the paper is 0·04 g. Assuming the paper and tobacco produce the same quantitites of 3,4-benzpyrene when smoked in a cigarette as they do when smouldered separately in the apparatus described here, the paper of a cigarette would produce 0·042 and the tobacco 0·122 γ. The total per cigarette is then 0·162 or for 400 cigarettes, 64·2 γ. This is in line with the results obtained with cigarettes smoked in the smoking machine (40 to 49·2), considering that $\frac{1}{4}$ to $\frac{1}{5}$ of the cigarettes are discarded as butts in the latter experiment.

* Since the preparation of this paper, a sample of 9-methylanthracene was obtained. The fluorescence of this compound is also very much different from that of 3,4-benzpyrene and can be used to differentiate between the two compounds.

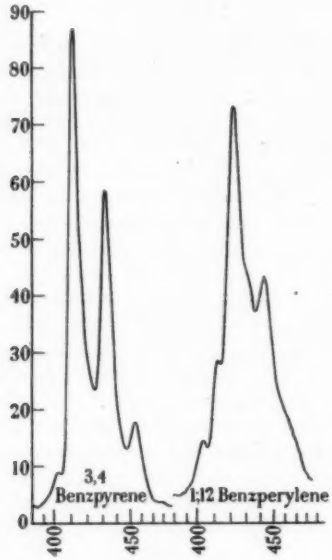

FIG. 11.—Fluorescence.

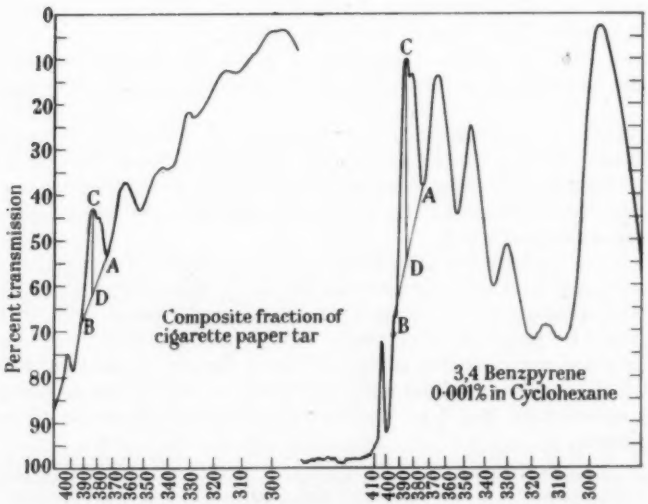

FIG. 12.—UV absorption.

The variation in 3,4-benzpyrene formation in the smoke of the different brands of cigars suggests the high value in the one brand may be a product of an additive used in this tobacco. The other brands show the expected similarity to tobacco.

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THE INHIBITION OF FORMATION OF 3,4-BENZPYRENE IN CIGARETTE SMOKE*

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In a previous article (Cardon *et al.*, 1956) 3,4-benzpyrene was reported in the tars from the smoke of cigarette paper, tobacco, and cigarettes. Considering the possibility that cigarette smoke may be carcinogenic, as has been suggested in recent medical publications, it was considered of value to attempt the elimination of this well-known carcinogen from cigarette smoke.

As reported previously, cigarette paper produces more 3,4-benzpyrene than does tobacco per weight of material smoked. Also, the detection of 3,4-benzpyrene is simpler in tars from paper than in tobacco or cigarette tars. Our initial effort was therefore to modify the composition of the paper, thereby affecting the combustion and combustion products so that less 3,4-benzpyrene would be present in the paper smoke.

Cigarette paper was treated with aqueous and non-aqueous solutions of various chemicals, smoked, and the smoke analyzed for indications of 3,4-benzpyrene. A rapid screening method involving one chromatographic column was used. Indications of the presence of 3,4-benzpyrene were obtained from the fluorescent spectrum; peak fluorescence at 410 and 432 mμ was considered to be due to 3,4-benzpyrene. The quantity of eluent and the intensity of this peak fluorescence was assumed to be a rough indication of the quantity present.

Using this method, many compounds were added to paper and the effect on 3,4-benzpyrene production noted. Concentrations of 5 to 15 per cent by weight of the paper were used. In general, substances which would be expected to produce acid vapors on heating in moist air, reduced the formation of total tar but left the 3,4-benzpyrene unaffected or somewhat increased. In this class are the halides of calcium, barium, magnesium, zinc, sodium, and potassium. Sulfates, nitrates, acetates, phosphates, and perchlorates did not have much effect though alkaline salts like sodium acetate and trisodium phosphate increased the production of fluorescent tars. Alcohols and organic acids were without effect.

Ammonium compounds of strong acids, i.e., phosphate, sulfate, sulfamate, and chloride, sharply reduced the production of 3,4-benzpyrene in cigarette paper tars. The nitrate was ineffective, as were ammonium salts of organic acids, presumably because ammonia is not produced by these materials when they are heated. Amines and aminoalcohols reduced benzpyrene formation but less so than the inorganic ammonium salts. The effect was roughly proportional to the "effective" ammonia content; thus, ammonium sulfamate had the same effect as an equal weight of ammonium sulfate or twice the weight of monoammonium phosphate.

* This paper was presented at a meeting of AAAS, Atlanta, Ga., Dec., 1955.

For more quantitative data on the 3,4-benzpyrene content of the paper tars, larger batches of paper were burned as described (Cardon *et al.*, 1956) and the separation technique carried through to solutions in which the ultraviolet absorption curves gave peaks adequate for the quantitative estimation. The tar of paper containing 4 per cent or more of a diammonium salt or its equivalent contained one part or less per 15 million of 3,4-benzpyrene by weight of the paper burned. A diammonium salt content of 7 per cent reduced the 3,4-benzpyrene content to less than one part per 30 million.

Diammonium salts in the paper were unstable and lost ammonia on standing, presumably due to the presence of calcium carbonate filler in the paper. Monoammonium salts were stable. Ammonium sulfamate, although monobasic, contains two effective ammonium groups and had the same inhibiting effect as ammonium sulfate but was stable.

Tobacco containing ammonium sulfamate similarly produced less benzpyrene. Cigarettes prepared from treated paper produced less than half the benzpyrene than did cigarettes prepared from the same tobacco (not a popular brand) and untreated, paper. As previously reported (Cardon *et al.*, 1956) the relative production of benzpyrene by the paper and tobacco is approximately 8 : 1, but since the paper is 1/25 the weight of the cigarette, the expected ratio of benzpyrene from the paper and tobacco is 1 : 3. The more than 50 per cent reduction in benzpyrene formation suggests the presence of ammonium salts in the paper reduces the benzpyrene production from the tobacco.

EXPERIMENTAL

Chemicals

The solvents used were c.p. or reagent grade. The chemicals tested were c.p. except where these were not available as in some of the organic materials like ethanolamines. Reagent and technical grade ammonium salts were used indiscriminately with identical results.

Procedure

1. *Paper*

The general technique for treating the cigarette paper with water soluble materials was to dip the paper from a standard commercial roll in an aqueous solution of the desired material and pass the wet paper on a stainless steel belt under infra-red lights to dry.

The paper was burned and the tars collected in acetone as previously described (Cardon, *et al.*, 1956). The acetone solution was shaken in a separatory funnel with cyclohexane and water. The water acetone layer was discarded and the cyclohexane solution washed free of acetone with more water and dried over anhydrous calcium chloride.

The cyclohexane solution was passed through a column of activated alumina 1-$\frac{1}{2}$ inch in diameter $\times$ 4 inches long, and the column eluted with benzene. The benzene eluents were examined for the 2-peak fluorescence spectrum characteristic of 3,4-benzpyrene in dilute mixtures. Paper, the tars of which gave eluents in which this type of fluorescence was comparable qualitatively to that produced by untreated paper, was not considered further. In those cases in which the 2-peak fluorescence spectrum was present in smaller amounts of solution and was of

lower intensity, larger batches of paper were burned and the separation technique for 3,4-benzpyrene carried through to ultra-violet absorption curves and a quantitative estimate of the 3,4-benzpyrene produced per gram of paper burned was made by the technique previously described.

Soluble sulfates, nitrates, and acetates of Ca, Mg, Ba, Na, K and Zn in the paper did not reduce the benzpyrene in the corresponding tars. The halides of Ca, Mg, Zn, and Ba reduced the acetone soluble material, non-volatile at 100° C, to about 20 per cent of that formed from untreated paper, but had no inhibiting action on the formation of 3,4-benzpyrene. Alkaline salts, that is sodium salts of phosphoric, boric, acetic, and benzoic acid, increased the amounts of material with high overall fluorescence and made the detection of 3,4-benzpyrene more difficult. Repeated chromatography nevertheless showed as much benzpyrene in these tars as in tars from untreated paper.

Ammonium salts of sulfuric, sulfamic, phosphoric, hydrochloric, persulfuric, and perchloric acids caused a sharp reduction in the benzpyrene content of the tars from the papers containing them. Ammonium nitrate and ammonium acetate, which do not produce ammonia when heated, had little inhibiting effect on the benzpyrene formation. These results are summarized in Table I.

TABLE I.—*Classes of Compounds Tested**

Type of compounds.	Example.	Per cent pickup.	Remarks.
Neutral salts	Potassium sulfate	10	Definite peaks at 410 and 432.
Organic salts	Sodium citrate	11	Definite peaks.
Organic amines	Triethanolamine	10	Low peaks.
Inorganic ammonia salts	Ammonium sulfate	7	Low fluorescence. No peaks.
Inorganic oxidizing agents	Potassium persulfate	8	Definite peaks.
Organic ammonium salts	Ammonium acetate	10	Definite peaks.
Aromatic salts	Sodium benzoate	8	Definite peaks.
Inorganic acid salts	Sodium bisulfate	8·2	Definite peaks.
Dehydrating agents	Calcium chloride	15·5	Very little tars but high peaks.
Aromatic oxidizing agents	Benzoyl peroxide	9	Burns fast. Definite peaks.
Inorganic alkalies	Sodium carbonate	8	Definite peaks.
Inorganic amines	Sodium sulfamate	7	No peaks.
Complex amine salts	Nickel hexamine sulfate	10	Low tars. Low peaks.
Aromatic acids	Benzoic acid	5	Definite peaks.
Fire retardent	Boric acid	8	Low tar formation. High peaks.
Metallic salts	Sodium stannate	12	Low tars. Peaks.
Nitrogen aromatics	Pyridine	5	Peaks.
Hydroxy acids	Gluconic acid	10	Peaks in two eluents.
Fatty acid salts	Sodium lauryl sulfate	15	Peaks.
Miscellaneous	Mandelic acid	8	Definite peaks.
Miscellaneous	Chlorophyl	10	Definite peaks.

* 5 per cent solutions were used to treat the paper.

Storage tests on papers containing ammonium salts were run by periodically analyzing the paper for the ammonia content. This was done by adding a weighed sample of the paper to a 10 per cent solution of sodium hydroxide in a flask fitted with a liquid trap and distilling over the ammonia into an aqueous boric acid solution. The distillate was titrated with standard acid. To confirm the analysis,

Kjeldahl digestions of the paper were run on some samples. The results are summarized in Table II. The monoammonium salts are stable, whereas the diammonium salts lose some ammonia on standing, perhaps due to the action of calcium carbonate present as filler in the cigarette paper.

TABLE II.—*Loss of NH_3 From Treated Cigarette Paper*

Compound.	Date: 11.xi.54. % NH_3.	22.xi.54. % NH_3.	6.xii.54. % NH_3.	25.i.55. % NH_3.	11.ii.55. % NH_3.
Ammonium sulfate	1·85	1·21	1·13	—	—
Diammonium phosphate	1·45	1·28	1·12	—	—
Monoammonium phosphate	1·26	1·25	1·15	—	—
Ammonium sulfamate	0·89	0·95	0·88	0·88	0·88 and 1·78*
Ammonium persulfate	—	1.63	—	—	1·57†

* Kjeldahl digestion.
† Turns brown on ageing.

The inhibiting effect was roughly proportional to the ammonia content of the paper. This can be seen qualitatively in Fig. 1. Thus, 3 per cent ammonium sulfate is more effective than 1 per cent ammonium sulfate and 5 per cent diammonium phosphate is more effective than 3 per cent diammonium phosphate.

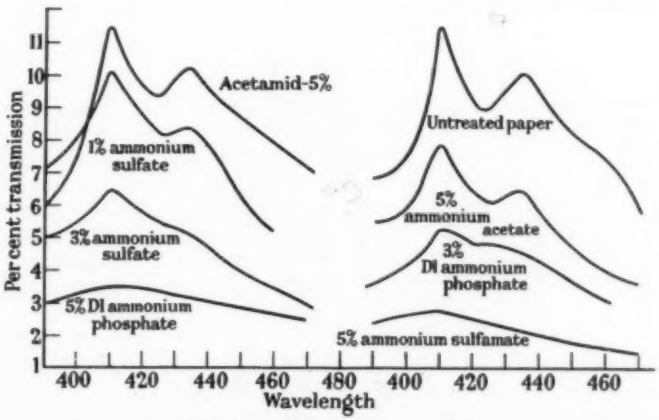

FIG. 1.—Comparative inhibitory effects of various substances on 3,4-benzpyrene.

Because of its stability and relatively high ammonia content, ammonium sulfamate was studied more extensively as an additive to cigarette paper. Papers containing various percentages of ammonium sulfamate were burned and the quantities of 3,4-benzpyrene in the tars were determined. The results are graphically shown in Fig. 2. In determining the percent reduction, a figure of one part benzpyrene per million of paper was used for untreated paper which is an average of a number of determinations. The figure for the reduction effected by the 6·7

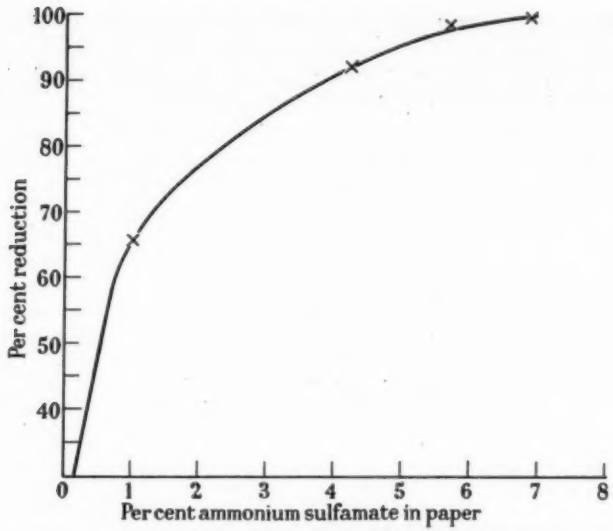

FIG. 2.—Reduction of 3,4-benzpyrene by ammonium sulfamate.

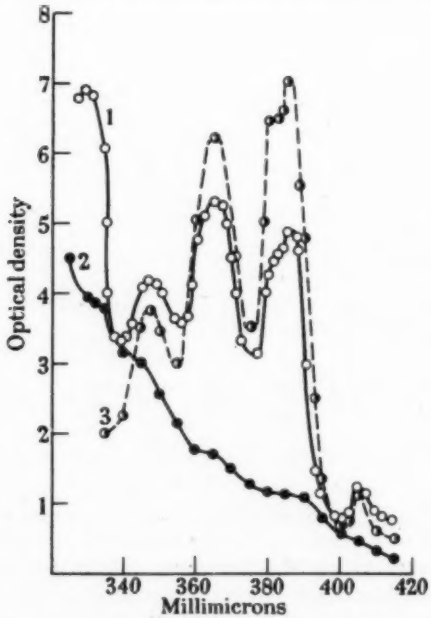

FIG. 3.—Absorption curves of the 3,4-benzpyrene from 300 g. of regular paper and from 1000 g. of paper containing 6·7 per cent ammonium sulfamate. 1. Untreated cigarette paper. 2. Treated cigarette paper. 3. 3,4-Benzpyrene (·001 per cent in isopropyl ether).

per cent paper is an estimate, as the absorption peak at 386 mμ was too low to make an accurate calculation.

Fig. 3 shows absorption curves of the 3,4-benzpyrene from 300 g. of regular paper and from 1000 g. of paper containing 6·7 per cent ammonium sulfamate.

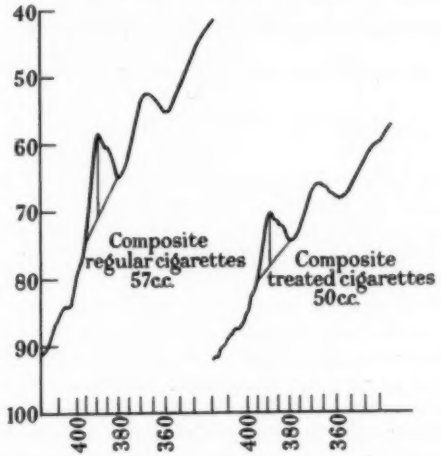

FIG. 4.—Absorption curves from an experiment comparing 3,4-benzpyrene production by regular and treated cigarettes.

2. *Tobacco*

Cigarette tobacco (1 lb.) was stirred in a solution of 9 g. of ammonium sulfamate in 50 ml. of water. The tobacco absorbed all of the solution and was then dried in air at room temperature. The dry tobacco was burned and the 3,4-benzpyrene content of the condensed tars determined by previously described techniques (Cardon, *et al.*, 1956). Approximately 1 part of 3,4-benzpyrene in 40 million parts of tobacco was found. Untreated tobacco of the same brand gave 1 part of 3,4-benzpyrene per 8 million parts of tobacco. A reduction of 80 per cent of 3,4-benzpyrene formed in the tobacco tars was thus achieved by the treatment.

3. *Cigarettes*

Cigarettes were made using paper containing 4·25 per cent ammonium sulfamate. Four hundred of these cigarettes were smoked in a smoke sampling apparatus and the tars were analyzed for 3,4-benzpyrene by previously reported techniques. The quantity of 3,4-benzpyrene formed was compared to that formed from 400 cigarettes made from the same tobacco and untreated paper. Seventy γ of 3,4-benzpyrene were found in the tars of cigarettes with untreated paper and 28 from those made with treated paper. A repeat experiment gave 67 γ and 31 γ, respectively. Fig. 4 shows absorption curves for the latter experiment.

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SOME PHENOLIC CONSTITUENTS OF CIGARETTE SMOKE

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PHENOLS have long been recognised as constituents of tobacco smoke although the conditions employed in obtaining the samples were in some cases very unlike those occurring in normal human smoking. Phenol, *o*-cresol, *m*-cresol, catechol, guaiacol and less well defined constituents designated as polyphenols, phenolic acids and creosote have been identified and in some cases determined. Of this earlier work the most significant was that in which phenol, *o*-cresol and guaiacol were separated and determined by paper chromatography of the azo-dyes formed with *p*-nitraniline (Rayburn, Harlan and Hanmer, 1953). A well-designed smoking machine was employed to produce the smoke condensate in this investigation.

The new method for determination of phenols (Commins and Lindsey, 1956) is especially suitable to separations on a microgram scale and depends upon the quantitative conversion of phenols into their methyl ethers by dimethyl sulphate in the presence of alkali, followed by chromatographic separation in cyclohexane solution on alumina columns. The detection and determination of the ethers in successive eluates is effected by the recognition of characteristic absorption peaks in the ultra-violet spectra and the measurement of peak heights.

EXPERIMENTAL

The mainstream smoke condensate from 50 cigarettes of a well-known brand sold in this country was prepared mechanically by the smoking method previously described (Cooper and Lindsey, 1955) and the product dissolved in absolute ethyl alcohol. The red solution was diluted somewhat with water and the mixture extracted several times with chloroform containing 5 per cent of ethyl alcohol. The phenols were then transferred into aqueous 2N sodium hydroxide solution by repeated extraction and the alkaline solution methylated by heating under reflux with excess of dimethyl sulphate for 3 hours. Finally, the methyl ethers were transferred to cyclohexane by repeated extraction and the volume adjusted to 10 ml. Aliquot portions of this solution were analysed by chromatography followed by ultra-violet spectrophotometry.

The phenyl- and the three cresyl-ethers were determined in 0·4 ml. of the solution by separation on a 10 cm. column of alumina de-activated with one per cent of water.

The dimethoxybenzenes were determined in 0·3 ml. of the solution by separation on a 5 cm. column of alumina de-activated with 5 per cent of water and the same type of alumina was used for the naphthyl ethers by using 3·0 ml. of the solution on a 7·5 cm. column. A second chromatographic separation was necessary to determine both the naphthols.

Finally the quantities of each ether in successive fractions were determined by measuring peak heights obtained with the Unicam SP 500 absorption spectrophotometer. The results, expressed as micrograms of the phenol in the smoke of one cigarette are shown in Table I.

TABLE I.—*Phenols present in Cigarette Smoke, expressed in Micrograms per Cigarette*

Phenol	123
o-Cresol	22
m-Cresol	18
p-Cresol	40
1-Naphthol	0·27
2-Naphthol	0·54
Catechol	61
Resorcinol	8
Quinol	83

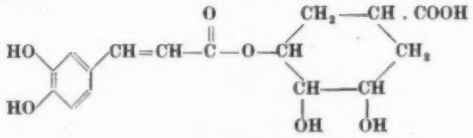
Note.—The monomethyl ethers of the dihydroxybenzenes, if present, are recorded as dihydroxybenzenes. Approximately 30 micrograms of guaiacol are present in the smoke from one cigarette and this is recorded in the figure for catechol above. Similarly the mono-ethers of resorcinol and quinol may be present.

DISCUSSION

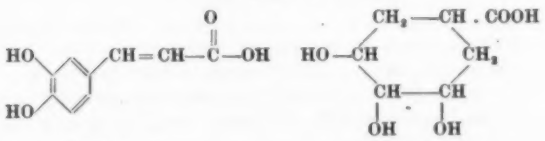
By this method the identification and determination has been accomplished of a number of phenols not previously reported present in cigarette smoke. In this category are the compounds *p*-cresol, 1-naphthol, 2-naphthol, resorcinol and quinol (the latter two possibly present in part as their mono methyl ethers).

All the phenolic constituents of tobacco smoke are of interest since it has been reported that some phenols are carcinogenic (Rusch, 1955). Of the phenols identified and listed in Table 1, 2-naphthol has been shown to produce sarcomas (Shear and Stewart, 1941), and recently Wynder in a private communication has stated that a phenol-containing fraction of cigarette smoke produces malignant growths when painted on mouse skin.

The origin of phenolic compounds in tobacco smoke has been previously discussed. Molinari (1936) was of the opinion that phenols originated from carbohydrates by pyrolytic processes in the smouldering tobacco and quoted an experiment in which similar componds were formed from pure cellulose. Wenusch (1939) assumed that the more complicated phenolic substances normally present in the cured tobacco leaf such as chlorogenic acid or its component parts, quinic acid and caffeic acid, break down upon heating into simpler phenols such as catechol or guaiacol.

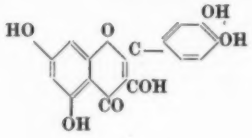


Chlorogenic acid

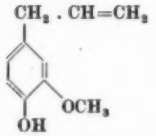


Caffeic acid Quinic acid

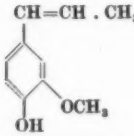
Many other phenols are present in the cured tobacco leaf and these compounds could well be distilled or steam distilled as the smouldering zone advances during smoking. Thus quercitin and rutin, its rhamno glucoside, as well as the simpler phenols, eugenol and *iso*-eugenol have all been found in tobacco and could give rise to catechol or guaiacol upon pyrolysis.



Quercitin



Eugenol



Iso-eugenol

A further and extraneous origin of phenols in tobacco is the " fire curing " process in which the leaves are hung in wood smoke for a long period, sometimes for several weeks. Many smoke constituents are thus deposited upon the leaves, and the phenol content of such material may be as much as fifteen times as great as in an air-cured product. It is unlikely that the Virginia cigarettes used in the present investigation contained any fire-cured material, as the method is used for other types of tobacco product. It is certainly established that, whatever may be the original compounds from which the phenols are formed, they are always present in the pyrolytic or partial combustion products of vegetable matter.

SUMMARY

1. A number of phenols has been identified and determined in cigarette smoke. The compounds so analysed were phenol, *o*-cresol, *m*-cresol, *p*-cresol, 1-naphthol, 2-naphthol, catechol, resorcinol and quinol. Of the three latter compounds, catechol is present partially as its monomethyl ether, guaiacol, and it is possible that the others may be similarly present.

2. *p*-Cresol, 1-naphthol, 2-naphthol, resorcinol, and quinol are reported present for the first time in tobacco smoke.

3. The results may be of significance as some phenols have elsewhere been described as carcinogenic.

The authors wish to thank the Medical Research Council for supporting this investigation.

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EXPERIMENTAL PRODUCTION OF CANCER WITH CIGARETTE TAR: STRAIN DIFFERENCES

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SINCE it was shown recently that a significant number of skin cancers could be produced in mice upon the application of condensed cigarette smoke it has become of interest to search for possible differences in tumour suceptibility among different strains of mice (Wynder, Graham and Croninger, 1953, 1955).

METHOD

A cigarette smoking machine was designed which in principle was identical to the one previously described excepting that it smokes 100 cigarettes at a time (Wynder, Graham and Croninger, 1953). It uses a 2-second puff every 18 seconds. The maximum combustion temperatures ranged between 850°–890° C. Regular size cigarettes of a popular brand were used. The resulting tar was stored, and at bi-weekly intervals prepared into 1 : 1 acetone-tar solution and applied to the backs of mice 3 times a week. An average of 65 mg. of tar was applied to the mice during each painting except during the first month when a smaller amount was used so as to desensitize the mice to the nicotine content of the tar. The study was conducted with 40 Swiss mice obtained from Millerton Farms in New York and 50 CAF_1 mice from the Jackson Memorial Laboratories in Bar Harbor, Maine. The animals were about 12 weeks old at the start of the experiment. The animals were shaved with an electric clipper, Oster ‡‡4, at the onset of the experiment and whenever hair growth reoccured. The entire back of the animals was painted using a ‡‡5 camel's hair brush.

RESULTS

A substantial number of carcinomas and papillomas have been produced. (Table I). The data show the survival rates for the CAF_1 mice to be significantly greater than for the Swiss mice. For instance, at the end of one year only 48 per cent of the Swiss mice were still alive as compared to 80 per cent of the CAF_1 mice.

Of perhaps greater practical significance, however, is the fact that the Swiss mice used in this experiment are more susceptible to cancer formation than the CAF_1 mice. This is indicated both by the earlier tumor formation among the Swiss mice as well as by the greater number of cancers observed. Thus, the first papilloma was obtained among the Swiss mice four months earlier than the CAF_1, whereas the first cancer was observed 6 months earlier. The per cent of tumors in the two strains in later months is difficult to compare if one considers the number of animals at the start of the experiment because of the higher mortality

TABLE I.—*Survival Rate, Papilloma and Cancer Formation Among 50 CAF_1 (Jackson) and 40 Swiss (Millerton) Painted with Cigarette Tar**

Month.	Survival (%). CAF_1 (50 mice).	Survival (%). Swiss (40 mice).	Papilloma (%). CAF_1.	Papilloma (%). Swiss.	Cancer (%). CAF_1.	Cancer (%). Swiss.
1	90	100	—	—	—	—
4	82	87	—	5	—	—
6	82	80	—	13	—	—
8	82	75	2	30	—	3
10	80	58	18	33	—	8
12	80	48	28	35	—	8
14	74	35	32	35	2	18
16	46	15	38	40	4	25
18	46	8	42	43	8	30
20	24	3	46	45	10	35
22	8	0	46	45	12	35
24	2	0	46	45	12	35

* The per cent of tumors is based upon the initial number of mice used and represents the number of mice which developed tumors.

rate among the Swiss mice. Thus, less than 10 per cent of the Swiss mice were still alive at 18 months while nearly half of the CAF_1 mice were still surviving. The higher mortality of the Swiss mice is apparent even if one considers that it is in part due to death from cancer.

The formation of benign ulcers in both strains is about the same, an observation not confirmed by subsequent experiments with tar fractions which suggest a higher susceptibility for ulceration among CAF_1 mice.

Among CAF_1 mice painted with acetone solution and those kept in our laboratory we have observed no tumors. Among 80 Swiss Millerton mice kept for 18 months without any type of skin application one papilloma developed.

At the end of the experiment 45 per cent of the Swiss mice had developed papillomas and 35 per cent cancers. The greater susceptibility of Swiss mice to tumor formation was also observed by the fact that they tend to develop more multiple papillomas than CAF_1 mice. These observations have been confirmed in subsequent experiments.

DISCUSSION

Methodology: During the past few years, several investigators have reported that they could not repeat the results describing significant cancer formation upon the application of cigarette tar. The reason for this difference may well be variances in method. For instance, a British investigator who reported negative results never shaved the mice, applied tar only twice a week and, rather than painting the whole back dipped the tar with a glass rod into a small area at the nape of the neck (Passey, personal communication). While there is no objection to this or any other mode of application we believe that if anyone claims to repeat an experiment it should be carried out as described, subsequent to which any variations may be used that the experiment desires. Sugiura (1956) by using the same cigarette tar as used in this study and following a similar technique of tar application obtained comparable results using Swiss (Rockland) mice. Of the 44 mice surviving one year 27 per cent developed cancer of the skin.

In addition to the importance of methodology in tar application the methods employed in the collection of tar are of obvious additional significance. The method of smoking which includes the type of cigarette used, the puff volume, the length to which the cigarettes are smoked, the manner in which the smoke is condensed and the way in which the tar-acetone solution is prepared are among the important variables which must be considered. The influence that variations in tar application and collection may have on the carcinogenic properties of cigarette tar are currently being investigated in this laboratory.

Strain variances.—The more susceptible a given strain to tumor formation, the shorter the latent period and the earlier results can be expected. This is a significant advance, particularly, if one tests a large number of compounds, the results of which must be awaited before additional compounds and fractionation schemes can be tested. The present experiment indicates that by using Swiss mice the latent period is significantly reduced as compared to CAF_1 mice, a decrease which applies not only to earlier papilloma but also to earlier cancer formation. The greater mortality rate among the Swiss mice does not occur early enough to interfere with significant tumor formation. The results indicate that the final number of papilloma obtained with CAF_1 mice does not vary significantly from that of the Swiss mice but they appear much later. The total cancer formation varies significantly even at the end showing a significantly greater resistance to skin cancer formation among CAF_1 mice.

In current experiments at this laboratory Swiss mice continue to develop tumors consistently earlier than the CAF_1 mice. It is for this reason that the Swiss mice prove valuable though because of the importance of the tobacco research program we have continued to use both strains.

SUMMARY AND CONCLUSIONS

1. A significant number of papillomas and cancers have been produced in two strains of mice by applying condensed cigarette tar to the skin, thus confirming previous studies.

2. Swiss (Millerton) mice are significantly more susceptible as far as papilloma and cancer formation is concerned upon the application of condensed cigarette tar than CAF_1 (Jackson) mice.

3. The significance of animal research in tobacco tar, to stress again, does not lie in the fact that they prove that smoking causes cancers in man. This proof rests entirely upon human clinical, statistical and pathological data. The significance of the animal experiment in this field is to help in the identification of specific carcinogens for a particular animal or strain of animal. In this respect the use of a susceptible animal is of great value because by decreasing the latent period of tumor formation one can observe results more quickly. It is along this line, as well as to re-emphasize the importance of methodology in this program, that this communication has been presented.

This study has been carried out under a grant of the American Cancer Society.

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THE EFFECT OF 3–4-BENZPYRENE ON HUMAN FOETAL LUNG GROWN *IN VITRO*

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Received for publication May 26, 1956.

EXPERIMENTAL induction of lung cancer in animals by distant or direct application of coal tar or carcinogenetic hydrocarbons has been reported by various authors. Murphy and Sturm (1925) obtained an increase in the incidence of lung cancer in mice painted with coal tar. This result was confirmed by Schabad and his co-workers (1930, 1935*a*, 1935*b*) who also observed development of lung tumours in mice after subcutaneous injection of 1-2-5-6-dibenzanthracene. Andervont obtained similar results (1934, 1935, 1937, 1938, 1940) after injection of dibenzanthracene and also induced adenocarcinomas, squamous cell carcinomas and sarcomas of the lung in mice by direct application of the carcinogen. Kimura (1923) and Garschin and Pigaleff (1931) obtained lung tumours in guinea pigs and rabbits by forcing coal tar into bronchi and parenchyma. Andervont and Shimkin (1940) and Shimkin and McClelland (1949) report the induction of lung cancer in mice after injection of 3–4-benzpyrene and 20 methylcholanthrene, and Horning (1950) obtained a similar result in lung fragments implanted into mice subcutaneously with methylcholanthrene crystals.

Squamous metaplasia of the bronchial epithelium in rats was observed by Thornton and Adams (1944) eight weeks after application of 3–4-benzpyrene, but no tumours appeared.

Recently Cooper, Lindsey and Waller (1954) and Cooper and Lindsey (1955) demonstrated the presence of 3–4-benzpyrene in condensate obtained from tobacco and cigarette paper under conditions similar to those of human smoking. The authors found that the condensate from 500 cigarettes contained approximately 4 μg. of 3–4-benzpyrene.

Their result raised the question of whether this carcinogen can be held responsible for the increase in lung cancer of cigarette smokers. The survey of the literature shows that lung tumours have been induced in various animal species by carcinogenic hydrocarbons, but it is well known that the sensitivity to carcinogens differs widely in different species and with different methods of application. So far, there is no experimental proof that *human* lung is susceptible to benzpyrene.

This study is an attempt to obtain information on this point by means of the organ culture technique, since earlier work in which the same method was used has shown that 20-methylcholanthrene directly induces changes of a precancerous nature in mouse prostate glands *in vitro* (Lasnitzki, 1951, 1954, 1955).

In the present experiments the direct effect of 3 concentrations of 3–4-benzpyrene on human foetal lung grown in culture is described and analysed. In all, 176 explants from five foetuses were studied.

* Sir Halley Stewart Fellow.

MATERIAL AND TECHNIQUE

The material was obtained from 3–5 months old male foetuses after a surgical termination of the pregnancy. At this stage (Fig. 1), the lung consists of bronchioli and pneumonomeres embedded in cellular connective tissue. The lungs were removed aseptically, cut into fragments of approximately 2–3 mm, and 6–8 pieces were explanted in each culture vessel.

The watchglass technique (Fell and Robison, 1929) was used; the characteristic histological structure of tissues grown by this method is preserved whereas with most other types of culture only outgrowth of unorganised fibroblasts or epithelial cells is obtained. The tissue is placed on the surface of a plasma clot contained in a watchglass; the watchglass is enclosed in a petri dish and rests on a layer of moist sterile cotton wool which prevents evaporation.

In the present experiments the medium consisted of a mixture of chick plasma, horse serum and chick embryo extract in a proportion of 2 : 1 : 2. Penicillin was added to all cultures in a concentration of 300 i.u. per ml. of medium.

A 0·4 per cent solution of 3–4-benzpyrene in acetone was shaken into horse serum and this suspension was added to the medium before clotting. The doses employed were 1, 4 and 6 μg. per ml. of medium. The concentration of acetone in the medium did not exceed 0·4 per cent, corresponding amounts of acetone in horse serum were added to all control cultures and appeared to have no harmful effect on the tissue.

Three sets of experiments were made:

(1) Application of 1 μg./ml. for 4 weeks.
(2) Application of 4 μg./ml. for 4 weeks.
(3) Application of 6 μg./ml. for 2 weeks, followed by 4 μg./ml. for 2 weeks.

The explants were transferred to a fresh clot and fresh carcinogen every 3 to 4 days and fixed in 3 per cent acetic Zenker's solution after one, two, three and four weeks' treatment. From six to fifteen pairs of experimental and control cultures were fixed after each of these intervals. They were serially sectioned and stained with haematoxylin and eosin, by the periodic acid Schiff technique or with a modified Azan stain in which carmalum had been substituted for azocarmine.

RESULTS

Control Explants

Living explants

Two to three days after explantation or transfer, the lung fragments became surrounded by a luxuriant out-growth, in several planes, of clear translucent structures (Fig. 2), the bronchioli, many of which branched into pneumonomeres, while unorganised fibroblasts and macrophages wandered out from the periphery of the explant. There was considerable liquefaction and by the fourth day many explants had become surrounded by a pool of liquid.

Histological structure

Sections of lung cultures resembled the original tissue and showed bronchioli and pneumonomeres embedded in cellular connective tissue (Fig. 3). There was,

however, more branching and new formation of epithelial structures than *in vivo*. They were usually lined with one layer of secretory epithelium but occasionally reserve cells were seen ; the lining epithelium consisted of cuboidal or cylindrical cells with an oval nucleus and a conspicuous cytoplasmic vacuole facing the basal membrane (Fig. 4). In the bronchioli cilia were sometimes present, and cell divisions were frequent both among the bronchiolar epithelium and the connective tissue. Cartilage appeared in many cultures after two to three weeks' growth.

Effects of 3–4-Benzpyrene

Living explants

Cultures grown in the presence of 1 and 4 μg of benzpyrene showed outgrowth of translucent bronchioli and pneumonomeres similar to those seen in the controls. In explants treated with 6 μg. these structures often lost their transparency and prolongation of treatment beyond two weeks sometimes produced cultures which appeared opaque and had ragged edges.

EXPLANATION OF PLATES

FIG. 1, 2, 3, 4.—Untreated.

FIG. 1.—Human lung of a 3½ months old foetus before explantation. Haematoxylin-eosin. × 130.

FIG. 2.—Living lung culture after two weeks' growth *in vitro* ; three days after transfer. Note the spreading of bronchioli and pneumonomeres at the periphery of the explant. × 25.

FIG. 3.—Section through a similar culture after 3 weeks' growth *in vitro*. Haematoxylin-eosin. × 130.

FIG. 4.—Bronchiolus and pneumonomere in a control culture at higher magnification. Azan substitute. × 320.

FIG. 5, 6, 7, 8.—Cultures treated with 3-4 benzpyrene.

FIG. 5.—Culture grown for four weeks with 1 μg. of 3-4-benzpyrene. Note heaping of cells in the bronchiolar epithelium. Azan substitute. × 200.

FIG. 6.—Culture grown for four weeks with 4 μg. of 3-4-benzpyrene showing hyperplastic bronchioli and pneumonomeres. Note the enlargement of the epithelial cells, and the paucity of the stroma. Haematoxylin-eosin. × 150.

FIG. 7.—Culture grown for two weeks with 6 μg. and for one week with 4 μg. of 3-4-benzpyrene showing an almost occluded bronchiolus. Note the extreme paucity of the stroma. Haematoxylin-eosin. × 280.

FIG. 8.—Culture grown for two weeks with 6 μg. of 3-4-benzpyrene showing increased formation of bronchioli and one hyperplastic bronchiolus. Haematoxylin-eosin. × 100.

FIG. 9, 10, 11, 12, 13.—Cultures treated with 3-4-benzpyrene.

FIG. 9.—Hyperplastic bronchiolus showing flattening and stratification of cells in a culture treated for four weeks with 1 μg. of 3-4-benzpyrene. Haematoxylin-eosin. × 450.

FIG. 10.—Hyperplastic bronchiolus in a culture treated for four weeks with 4 μg. of 3-4-benzpyrene showing crowded small cells lined by a layer of secretory epithelium. Haematoxylin-eosin. × 450.

FIG. 11.—Part of a hyperplastic bronchiolus in a similar culture showing enlargement of cells, abnormal mitosis and degeneration of the secretory elements. Haematoxylin-eosin. × 570.

FIG. 12.—Hyperplastic bronchiolus in a culture treated for two weeks with 6 μg. of 3-4-benzpyrene. Note the abundance of cilia. Haematoxylin-eosin. × 350.

FIG. 13.—Multipolar mitosis in a culture treated for four weeks with 4 μg. of 3-4-benzpyrene Haematoxylin-eosin. × 2500.

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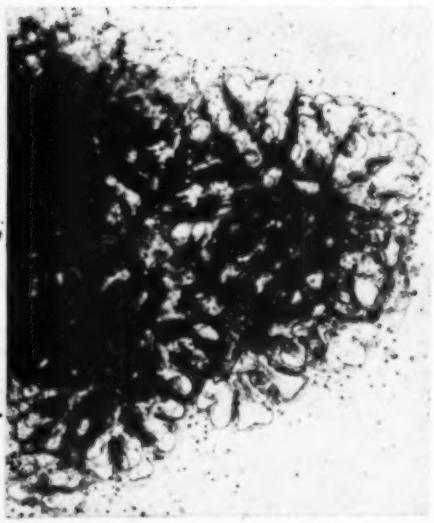

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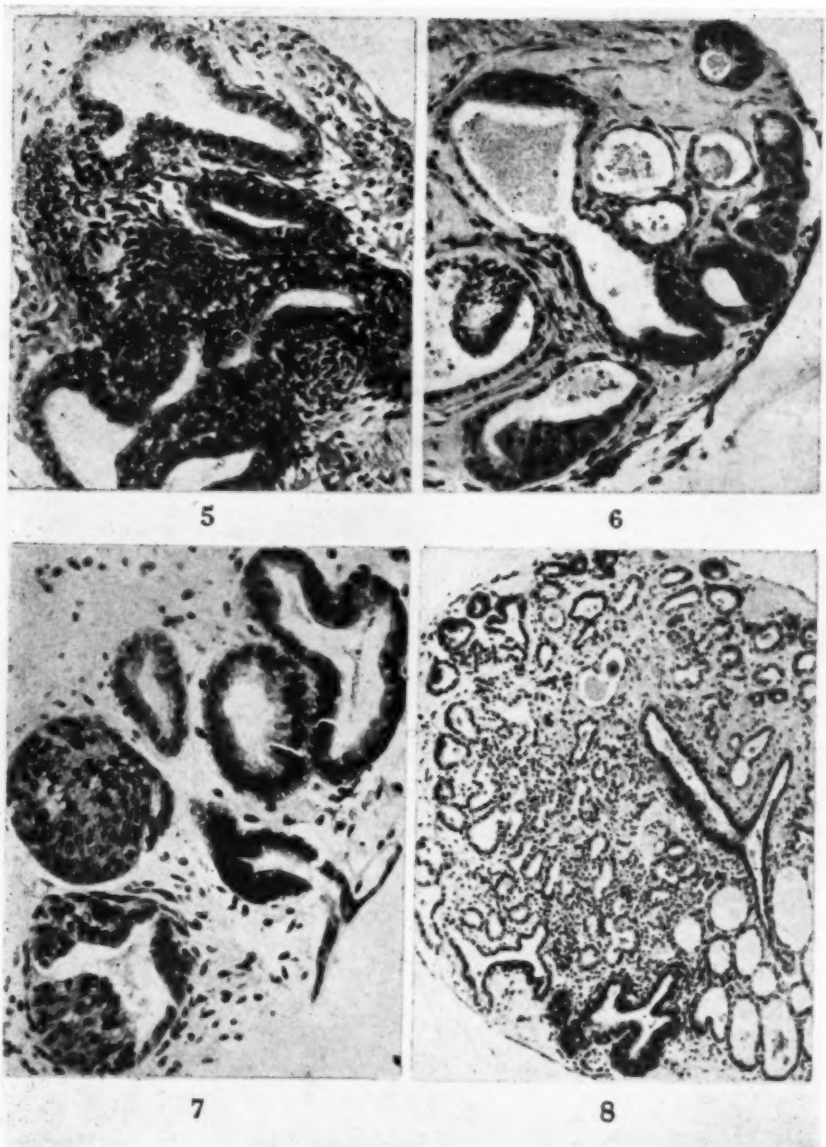

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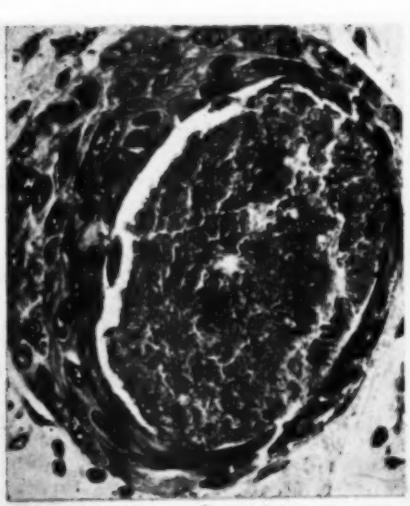

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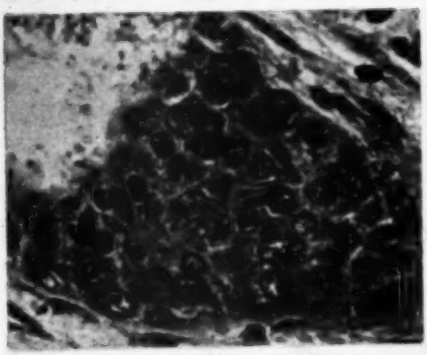

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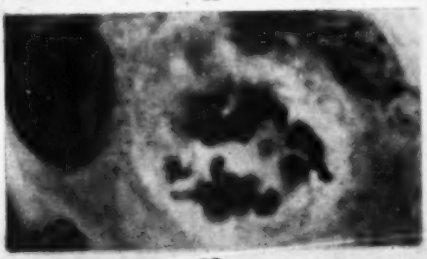

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The outgrowth of unorganised fibroblasts was inhibited by all three concentrations of the carcinogen ; it diminished with rising dose, and in cultures grown with 6 μg. it was very sparse or absent.

Histological structure

(1) 1μg. *per ml. of medium.*—Cultures fixed after one, two and three weeks' treatment were histologically identical with the controls. After four weeks, however, there was increased proliferation of the bronchiolar lining epithelium in five of six treated explants (Fig. 5). Three to four bronchioli or pneumonomeres per explant were involved and in these the number of cell layers had multiplied from the normal one to three or four. There were many normal cell divisions in both the unchanged and hyperplastic epithelium. In three hyperplastic explants the newly formed cells showed flattening and stratification accompanied by degeneration of the innermost cell layer (Fig. 9).

The development of cartilage was not impaired at this concentration but the rest of the connective tissue had become poorer in both cells and fibres and showed no mitosis.

(2) 4 μg. *per ml. of medium.*—After this concentration the first changes were seen after a fortnight's exposure when two out of seven treated explants fixed at this stage showed mild hyperplasia. After four weeks' treatment hyperplasia was present in eight out of nine explants examined. The number of hyperplastic foci were greater and the hyperplasia was more extensive (Fig. 6) than after the lower dose and the lumen was often partially or completely occluded. Cell multiplication usually began at one side of the bronchiolus whence the cells gradually invaded the cavity.

Stratification was not observed. The hyperplastic epithelium was either composed of crowded small round or oval cells which displayed frequent but normal divisions and were lined with a row of columnar secretory elements (Fig. 10), or consisted of irregular enlarged cells with one large or several smaller nuclei and abnormal mitosis while the innermost layer of secretory epithelium had disappeared (Fig. 11).

The abnormal cell divisions showed destruction of the spindle, and breakage, stickiness and degeneration of chromosomes. Cells in telophase were often seen in which one daughter nucleus appeared normal while the other contained only a few clumped chromosomes or chromatin granules. In other—probably the ancestors of the multinucleate cells mentioned above—multipolar arrangements of the chromosomes were observed (Fig. 13).

Secretion was increased in hyperplastic areas, and in sections stained by the periodic acid Schiff technique more PAS positive material was present in the lumen of hyperplastic pneumonomeres, particularly those in which the secretory epithelium was breaking down. Cilia which were only occasionally seen in control cultures were more numerous in hyperplastic bronchioli (Fig. 12).

The growth of the connective tissue was much inhibited at this concentration. Cell divisions were absent and there was a paucity of cells and fibres and no cartilage was found.

(3) 6 μg. *per ml. for a fortnight followed by* 4 μg. *per ml.*—After this concentration the incidence of hyperplasia was highest after a fortnight's exposure to the carcinogen but fell with continued treatment. Hyperplasia was present in 12 out of 15 explants treated for two weeks ; in 8 out of 13 treated for three weeks

(two weeks 6 μg., one week 4 μg.) and in only 4 out of 8 treated for four weeks (two weeks 6 μg., two weeks 4 μg.).

The histological changes were similar to those already described for explants treated with 4 μg., and consisted of increased proliferation of the bronchiolar epithelium with irregular enlargement of cells and abnormal mitosis (Fig. 7). In most explants exposed for two or three weeks hyperplastic foci were more numerous than after 4 μg. and the number of cell layers were greater. Stratification was also absent while secretion and ciliary development were increased. In addition to the production of individual hyperplastic foci a more general effect was seen, which resembled an adenomatous change, and consisted of increased branching and formation of pneumonomeres (Fig. 8).

The growth of the connective tissue was almost totally suppressed; the fibres were swollen and cartilage was absent. In some explants treated for 3 weeks or more, the epithelium began to atrophy and in places the bronchiolar epithelium was shed.

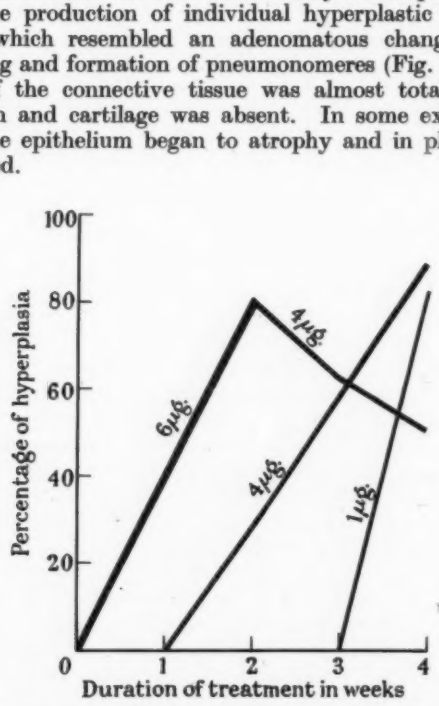

FIG. 14.—The incidence of hyperplasia in lung cultures treated with various doses of 3-4-benzpyrene. ——— 1 μg. ▬▬ 4 μg. ▬▬ 6 μg.

Fig. 14 gives the incidence of hyperplasia expressed as the percentage of explants showing hyperplasia against dose and duration of exposure. It is seen that the percentage of affected explants is similar after all three concentrations—80 to 89 per cent—but that the hyperplasia appears earlier with rising concentration. Thus with the lowest dose, 1 μg., the first changes occur after four weeks, with 4 μg. after two weeks, 28 per cent of the cultures being hyperplastic; after 6 μg. the greatest effect—80 per cent—is observed after two weeks, while prolonged exposure is followed by a fall to 62 per cent at three weeks and 50 per cent at four weeks.

DISCUSSION

The results show that 3–4-benzpyrene directly promotes the proliferation of the bronchiolar epithelium and causes an increase in the number of cell layers accompanied by irregular enlargement of cells and abnormal mitosis ; bronchioli and pneumonomeres are equally affected by the carcinogen independently of their size. At the same time the growth of the connective tissue and, at the higher concentrations the development of cartilage, are inhibited or suppressed.

The hyperplasia is not uniform but usually restricted to a limited number of pneumonomeres or bronchioli in each explant. Within the range of doses used with rising concentrations of the carcinogen, hyperplasia appeared sooner, was more pronounced and affected more bronchioli. The atrophy of the bronchiolar epithelium observed in some explants treated with the highest concentration for three or more weeks together with the lowered incidence of hyperplasia, indicate that at this dose level the carcinogen has a toxic effect which begins to mask its growth promoting action. Under the conditions of the experiment the optimum growth promoting dose seems to lie between 1 and 4 μg. of benzpyrene.

A comparison of the direct effects of 3–4-benzpyrene on foetal lung with those of 20-methylcholanthrene on mouse prostate glands, shows that the histological changes are similar. In both tissues epithelial hyperplasia occurred with an inhibition of stromal growth. After 20-methylcholanthrene the hyperplasia of the prostate was always followed by squamous metaplasia which increased with increasing dosage, while after benzpyrene flattening and stratification of cells in the lung was seen after the lowest concentration only.

A comparatively small dose of 20 methylcholanthrene—20 μg. applied over a period of 10 days—invoked a similar degree of hyperplasia in the mouse prostate as 84–105 μg. of benzpyrene given over a period of two or three weeks in the human lung. Pullinger (1941) using as criterion the induction of skin carcinomas in mice found that benzpyrene was approximately $\frac{2}{3}$ as active as methylcholanthrene. In these experiments the effectiveness of benzpyrene on the lung is only $\frac{1}{3}$ to $\frac{1}{4}$ of that found for methylcholanthrene on the mouse prostate gland, a result which indicates a lower sensitivity of the human lung. Although it is not certain whether this is due to a difference in the organ or species used it is of some interest as—so far—our knowledge of the action of carcinogenic hydrocarbons was mainly derived from experiments on rodents.

The epithelial hyperplasia of the foetal lung resembles that described for the initial stages of carcinogenesis in mouse skin after painting with 3–4-benzpyrene (Pullinger, 1940, 1941 ; Glücksmann, 1945) and may be considered precancerous. Whether the changes would ultimately lead to true malignancy under the more complex conditions *in vivo* remains to be decided. The finding that human lung is susceptible to 3–4-benzpyrene suggests, however, that this substance either alone or in conjunction with other compounds present in cigarette smoke cannot be excluded as a causative agent in human lung cancer.

SUMMARY

Human foetal lung was grown by the watchglass technique with and without addition of 3–4-benzpyrene in concentrations of 1, 4 and 6 μg./ml. of medium.

Explants grown in normal medium showed outgrowth of bronchioli and

pneumonomeres lined with one layer of columnar epithelium and formation of cartilage.

The addition of the carcinogen induced hyperplasia of the bronchiolar epithelium with partial or complete occlusion of the lumen. The newly formed cells showed irregular enlargement and abnormal mitosis. Treatment with 6 μg. produced, not only individual hyperplastic foci, but an increased general proliferation of new bronchioli and pneumonomeres resembling an adenomatous change.

The growth of the connective tissue was inhibited at all three concentrations.

The percentage of explants showing hyperplasia was 80–89 per cent for all three concentrations of benzpyrene, but with rising concentration of the carcinogen hyperplasia appeared earlier and the number of hyperplastic foci per explant and the degree of hyperplasia in them increased.

The changes were similar to, though less extensive than, those induced by 20-methylcholanthrene in mouse prostate glands *in vitro*.

I am greatly indebted to Mr. Oswald Lloyd, F.R.C.S., and Dr. Bruce Eton of Addenbrooke's Hospital, Cambridge, for their friendly co-operation in providing the foetuses used in these experiments. I would also like to thank Dr. Honor B. Fell, F.R.S., for advice and criticism in the preparation of this manuscript, Mr. George Lenney who made the microphotographs and Miss Marion Fakes for technical assistance.

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ON THE INFLUENCE OF CORTISONE ON THE EVOLUTION OF TUMOURAL GROWTH IN INTRASPLENIC OVARIAN GRAFTS IN TWO STRAINS OF MICE

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CORTISONE which counteracted local growth of a transplanted non-metastasizing adenocarcinoma in mice stimulated production of metastases of the same tumour (Agosin *et al.*, 1952). The finding of the Chilean group has been corroborated in work with other transplantable adenocarcinomata (Baserga and Shubik, 1954 ; Molamut, Spain, Gault and Kreisler, 1952). In the work with the transplantable Krebs-II adenocarcinoma cortisone produced widespread metastases beyond the lung filter, but no such result was obtained with Sarcoma 37 (Pomeroy, 1954). Likewise, cortisone did not affect the incidence, time of appearance or distribution of the anatomic sites of metastases of other transplantable tumours (Kaliss, Borges and Day, 1954 ; Martínez and Bittner, 1955).

It seemed of considerable interest to study the question whether cortisone may stimulate production of metastases of a tumour not transplanted but originating in the body under certain experimental conditions as in the hormonal imbalance established with intrasplenic ovarian grafts. As known from the work of Biskind and Biskind (1944) with rats, of Li and Gardner (1947) and many others with mice, and of our group with guinea-pigs (Lipschutz *et al.*, 1946 ; Iglesias, Mardones and Lipschutz, 1953 ; Mardones, Iglesias and Lipschutz, 1955) granulosa cell tumours, luteomata or mixed tumours originate under these experimental conditions (literature of work with the different species see Lipschutz, 1956). These tumours produce metastases which occur in mice (Furth and Sobel, 1947 ; Li, 1948) but are seemingly very rare in the rat and have been seen only once in the guinea-pig (Mardones, Iglesias and Lipschutz, 1955), almost five years after transplantation. In mice metastases occur sooner than that ; but in the strains with which we shall deal in the present paper metastases in the liver have never been found earlier than 404 days after transplantation. All these statements recommend the intrasplenic ovarian tumour as very suitable for the study of the influence cortisone may exert on production of metastases of tumours originating under experimental conditions.

Two strains kept in this laboratory have been used in the present work : C57bl and Balb A. Both were brought from the United States, kept for several years in the Institute of General Biology of the School of Medicine and from there they were introduced several years ago into our Institute. Operation was performed at the age of 32 to 120 days in females and at 43 to 76 days in males of C57bl ; age at operation varied in Balb A between 36 and 111 days. But age at operation was always uniform in the two comparative cortisone and non-cortisone groups. The animals were ovariectomized and one of the two ovaries, cut into various

pieces, was grafted into the spleen. The second ovary was grafted into the spleen of castrated males of the same litter or the same strain. About six months after grafting 2 or 4 pellets containing 40 per cent of cortisone acetate mixed with 60 per cent of cholesterol were implanted beneath the skin. The animals were necropsied 9 to 12 months after grafting the ovary and 3 to 6 months after the action of cortisone had begun. At necropsy three dimensions of the graft were measured with callipers ; the product is given as the " size " of the growth. These figures are quoted only to indicate the differences obtained in the various comparative groups of animals ; they are stultified by the fact that at necropsy cysts, large and small, mostly haemorrhagic, are unavoidably measured as part of the growth. A more exact comparison of size attained has been reached in another way which will be mentioned later. At necropsy a careful search was made for metastases, especially in the liver and lungs.

RESULTS

Metastases

We had at our disposal 55 animals not receiving cortisone in which ovarian tumours were produced, and 37 animals receiving cortisone in which ovarian tumours though of lesser size were found (Table II). In none of these 92 animals with ovarian tumours were hepatic metastases present at necropsy performed 302 to 407 days after grafting the ovary into the spleen.

Tiny nodules were found in the lung, especially at or near the pleura, in some of these animals, with greater incidence in Balb A. The nodules were as frequent in animals without as with cortisone. At microscopical examination the nodules appeared in some cases very suspect of being metastases of granulosa-cell tumours. But similar nodules are known to appear in the lung of mice spontaneously (Shimkin, 1955).

Absorption of cortisone was of 30 to 90 μg./day, during 4 to 6 months (Table I). Production of metastases, as evident from the present work, was not stimulated by the continuous absorption of these quantities of cortisone acetate. Absorption of the corticoid was calculated on the assumption of non-selective absorption (Fuenzalida, 1950 ; Fuenzalida and Lipschutz, 1953) ; should this assumption be wrong, the quantities of cortisone absorbed per day would be two and a half times those given above.

Metastases were found in the liver in 4 animals of strain Balb A not belonging to the groups classified in Tables I and II. Two of these animals were females which died at 404 and 431 days after grafting the ovary into the spleen ; 2 were males which died at 540 and 570 days. In all the 4 cases the ovarian growths were granulosa-cell tumours. None of these animals had received cortisone.

No metastases were found so far in any animal of the C57bl strain dying at 309 to 644 days.

Growth of the intrasplenic ovarian tumour

Full evidence was produced in our work that cortisone deeply interferes in the growth of the intrasplenic ovarian tumour. Results obtained are classified in Tables I and II.

There was a total of 66 animals not receiving cortisone. The ovary took in 57 animals (Table II) ; no remains of the ovarian graft were found in the remaining 9 animals. No less than 55 out of 57 animals with successful ovarian grafts had

ovarian tumours. In other words: when the ovary had taken in the spleen of these castrated females or males it was transformed into a tumour in more than 96 per cent of the cases. These results are fully in agreement with those of other authorities (Klein, 1953).

TABLE I.—95 *Animals with Intrasplenic Ovarian Grafts, 9 to 13 Months After Grafting.*

	(1) Strain and sex. C57bl	(2) No. of animals.	(3) Duration total days.	(4) Duration cortisone days.	(5) Cortisone μg./day.	(6) "Size"§ of growth average (cm.³).	(7) Significant difference σ.	(8) Minimum and maximum size (cm.³).
I	Female	19	305–377	0	0	1·95±0·5	2·0	0·020–7·34
II	„	13	302–374	119–180*	31–76	0·57±0·44		0·008–5·83
III	Male	9	315–378	0	0	1·69±0·6	1·6	0·220–5·67
IV	„	12	313–377	122–180**	30–84†	0·53±0·29		0·003–3·36
	Balb A							
V	Female	13	334–407	0	0	1·00±0·6	1·7	0·001–7·29
VI	„	8	331–372	121–203	41–94††	0·04±0·01		0·008–0·13
VII	Male	13	267–384	0	0	0·56±0·15	2·0	0·100–2·03
VIII	„	8	267–374	82–164	50–58†††	0·14±0·03		0·080–0·26

* One animal 66 days only. ** Three animals 41, 89 and 92 days only.
† Two animals with 102 and 121 μg./day. †† One animal with 113 μg./day. ††† One animal with 21 μg./day.
§ "Size" of growth: product of three dimensions as measured by callipers at necropsy, in centimetres.

TABLE II.—107 *Animals with Intrasplenic Ovarian Grafts.*

(12 animals included which were ommitted in Table I on account of very large cysts).

	Strain and sex. C57bl	(1) Cortis.	(2) Number total.	(3) With gran. c.t., or mixed.[1]	(4) With luteoma, or mixed.[2]	(5) Signs of degeneration.[3]	(6) No tumour or doubtful.[3]	Luteomatous reaction. (7) Number.[4]	Luteomatous reaction. (8) %.
I	Female	0	20	15	5	0	0	5	*25*
II	„	+	20	2	8	6 (5)	4 (3)	16	*80*
III	Male	0	10	8	2	0	0	2	*20*
IV	„	+	12	4*	1	5 (4)	2 (2)	7	*58*
	Balb A								
V	Female	0	14	6	7	(1**)	1 (1)	8	57
VI	„	+	8	1	3	0	4 (4)	7	88
VII	Male	0	13	3	9	0	1 (1)	10	77
VIII	„	+	10	2	4	1	3 (3)	7	70

[1] Granulosa-cell tumour, or mixed tumours but granulosa-cell nodules predominating.
[2] Luteomata, or mixed tumours but luteomatous tissue predominating.
[3] Refers only to animals in which ovarian structures were present.
( ) Animals with *cords or nodules of lutein cells*, often degenerated.
[4] Sum of colum (4) and ( ) of columns (5) and (6).
* Two very small ones.
** Partial degeneration of 1 of granulosa-cell tumours.

Taking of the ovary in animals receiving cortisone was as frequent as without cortisone. No remains of the ovary were found in 3 out of 53 animals receiving cortisone. But in 13 out of the total of 50 cases in which the ovary had taken, ovarian tumours were absent ; or, to be more explicit, we did not feel sure whether these tiny structures consisting of cords or nodules of lutein cells around follicular cysts still deserved to be classified as " tumours ". We shall deal more fully with this question in the next section.

The fundamental influence cortisone exerted on the evolution of the intrasplenic ovarian tumour was fully evident at necropsy and especially when tabulating results obtained by measuring with callipers the size of the grafts or more exactly, what seemed to be intrasplenic growths. The growths appeared to be smaller in the 4 cortisone groups than in the 4 corresponding groups without cortisone. However, the size of the growths varied enormously in all the groups. Thus, the first feeling was one of bewilderment. But when calculating the averages it was again found that the growths were smaller in all the 4 groups with cortisone than in the corresponding groups without cortisone. Since many of these grafts contained large cysts (compare Fig. 1, 2 and 3) it was thought that cysts might have stultified the results of tabulation. To avoid this error, animals in which the grafts contained large cysts, occupying more than half of the cut surface, as for instance Fig. 3, were omitted. Thus, only 95 out of 107 animals have been used for final tabulation (Table I). The results were again clear cut ; the average size of the grafts was, in the 4 cortisone groups, without any exception, considerably smaller than in the 4 corresponding groups without cortisone (see Column 6) notwithstanding the enormous variation in size in the groups themselves (Column 8). This explains why the significant difference (σ) between the non-cortisone and the cortisone groups was poor, not attaining even 2 in III/IV and V/VI (Table I).

EXPLANATION OF PLATES

FIG. 1.—Tumour of non-cortisone female C57bl (61). Solid granulosa-cell tumour, without cysts. × 3.

FIG. 2.—Tumour of non-cortisone female C57bl (61*a*). Predominantly granulosa-cell. Note large cysts. Omitted in Table I. × 3.

FIG. 3.—Tumour of non-cortisone female C57bl (17). Predominantly granulosa-cell ; *l*-luteomatous tissue. A haemorrhagic follicle also was present. Note large space occupied by loose connective tissue. × 3.

FIG. 4.—Largest tumour of non-cortisone females C57bl (65). × 3. (See also Fig. 9 and 10.)

FIG. 5.—Largest tumour of cortisone females C57bl (60). × 3. (See also Fig. 12.)

FIG. 8.—Almost solid part of predominantly granulosa-cell tumour of non-cortisone female C57bl (62). × 263.

FIG. 9.—Part of granulosa-cell tumour of Fig. 4 (65). Massive cords. × 263.

FIG. 10.—Part of the same granulosa-cell tumour (65). Annular disposition of cells. × 263.

FIG. 11.—Mixed tumour, predominantly luteoma ; non-cortisone female (41). × 270.

FIG. 12.—Granulosa-cell tumour of cortisone female (60). (Same animal as Fig. 5.) × 270.

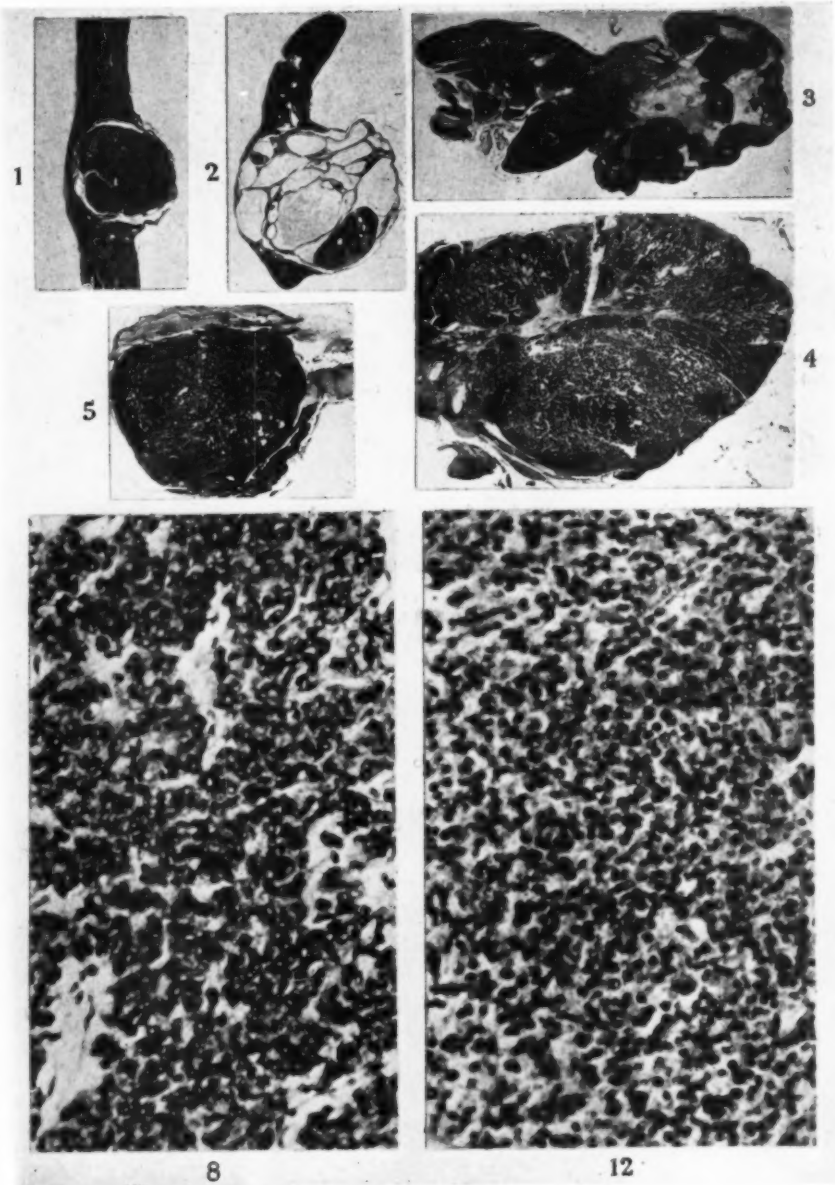
FIG. 13.—Mixed tumour, predominantly luteoma. Cortisone female (43). Part of the lutein cells greatly increased and hyalinized. × 270.

FIG. 14.—Small luteoma of cortisone female (39) on the surface of the spleen. Bottom—two lutein cysts ; top—nodule of luteinized cells. × 10.

FIG. 15.—Small luteomatous nodule of cortisone female (38). *Classified as non-tumourous or doubtful* (Column 6, Table II). Bottom—lutein cysts and lutein cords ; top—spleen with small cyst and lutein cords completely surrounded by the parenchyma of the spleen. Picture typical of the *guinea-pig* at about 6 to 10 months after grafting. × 37.

FIG. 16.—Wall of lutein cyst of Fig. 15. Lutein cells mostly degenerated. × 104.

FIG. 17.—Degenerated luteoma of cortisone female (15). Top—spleen ; bottom—leucocytic infiltration. × 36.



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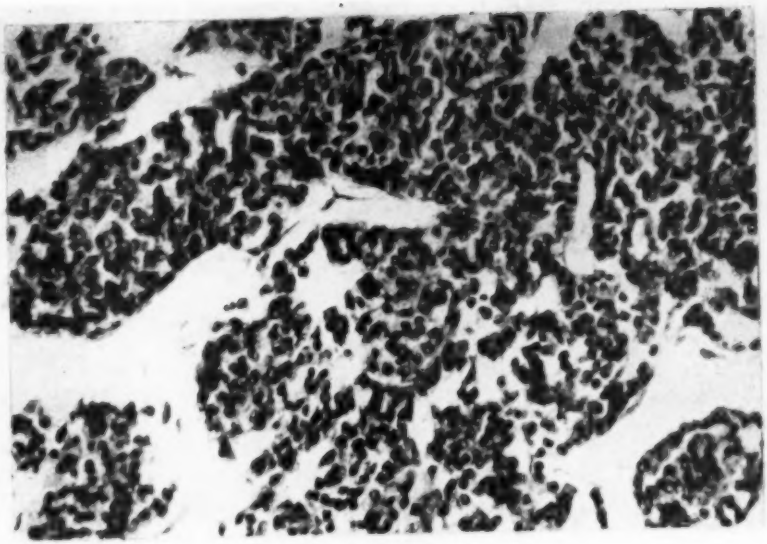

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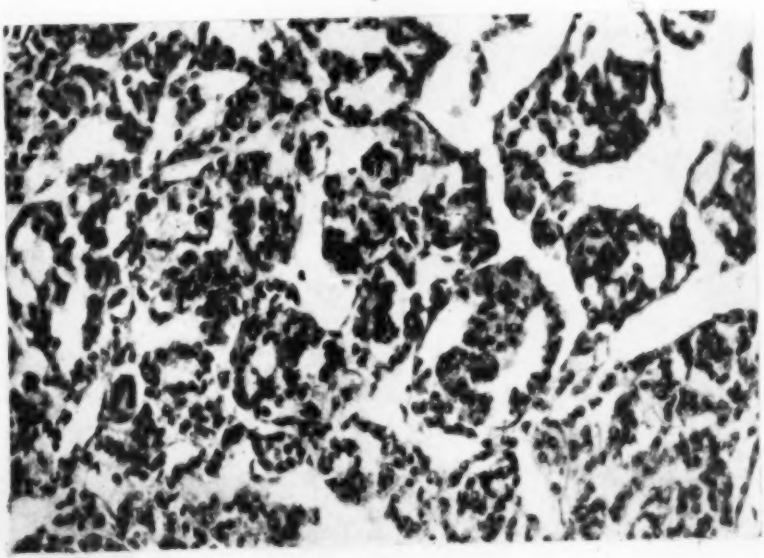

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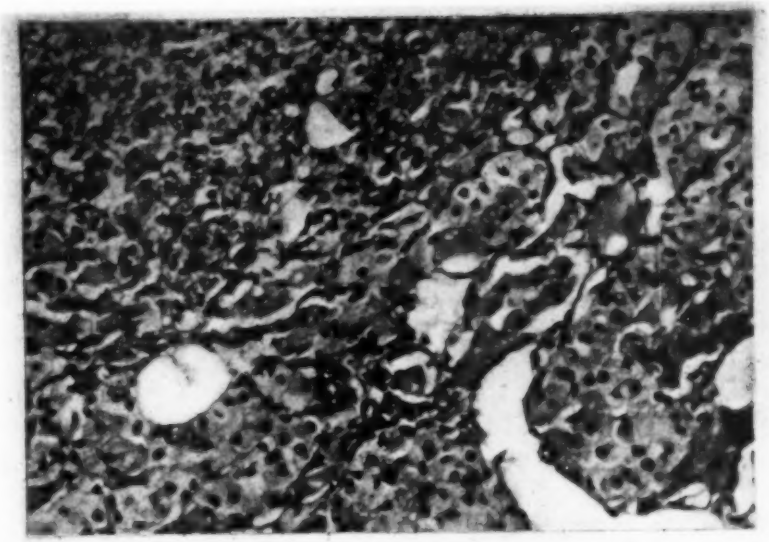

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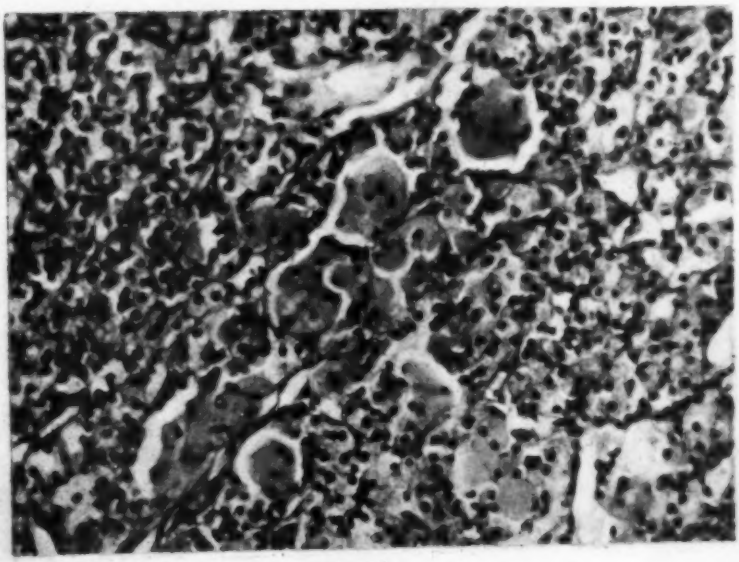

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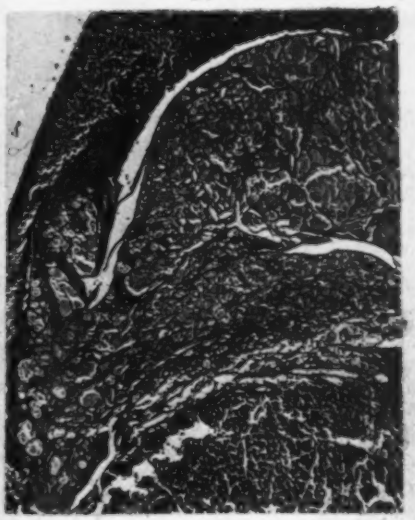

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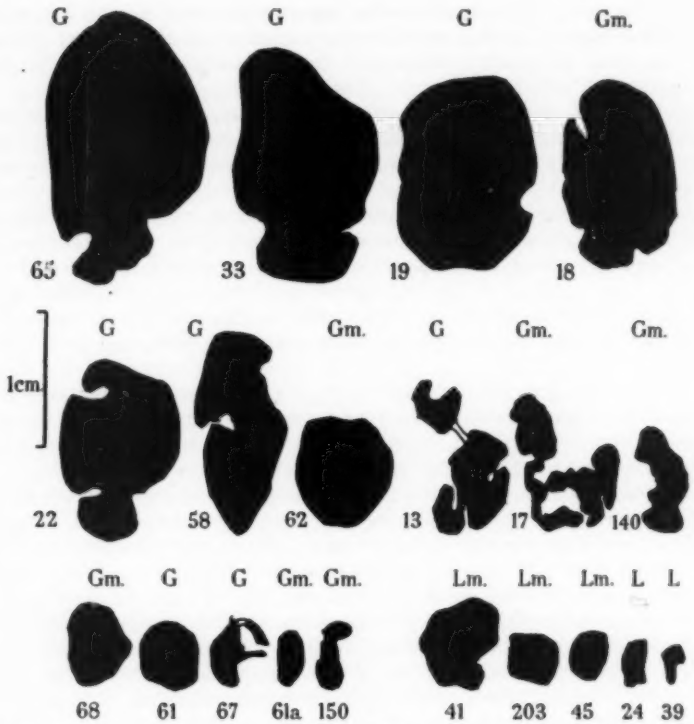

FIG. 6.—Surface occupied by granulosa-cell and luteomatous tissues in the microscopical slides of 20 non-cortisone females of C57bl (Group I in Table II). Cysts always omitted. G = granulosa-cell tumour ; Gm = mixed tumour but granulosa-cell tissue predominating ; L = luteoma ; Lm = mixed tumour but luteomatous tissue predominating.

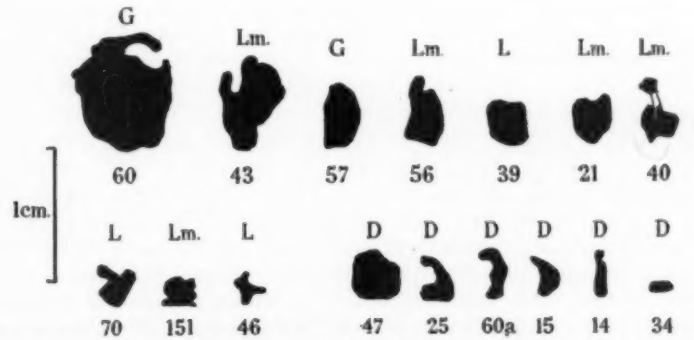

FIG. 7.—Surface occupied by the ovarian growth of 16 cortisone females of C57bl (Group II in Table II) ; 4 non-tumourous grafts have been omitted. D = degenerated.

There was another striking difference between the cortisone and non-cortisone groups: notwithstanding the bewildering variation in the size of the growth, maximal size was invariably smaller in the cortisone group than in the non-cortisone groups (Table I, Column 8: Fig. 4 and 5).

Of fundamental importance is also the fact already mentioned above, and to be discussed more fully in the next section, that several tiny grafts in the cortisone groups, especially in the Balb A strain, scarcely deserved to be classed as tumoural.

To avoid the error in the evaluation of the comparative size of the growths due to cysts the following procedure was adopted. The preparations of the 40 animals of Groups I and II (Table II) were photographed at $\times$ 3. The surface occupied by tumourous tissue (granulosa-cell and luteomatous) was then delineated with the control of the microscope (Fig. 6 and 7). The relation of the total surface of the tumourous tissue in the non-cortisone and cortisone Groups I and II was of 100 : 16.

Before proceeding to comparative microscopical observations in the cortisone and non-cortisone groups two interesting facts may be mentioned: the evolution of the ovarian tumour differed, apparently, according to the sex of the host and, certainly, according to the strain.

As seen from Column 6 of Table I the average size of the growths in the non-cortisone groups was greater in females than in males (compare I/III and V/VII). Also the maximal size attained in the females of the non-cortisone groups was larger than in males; this rule does not apply to those groups in which the evolution of the tumour was interfered with by cortisone (II/IV and VI/VIII). However, when discussing the comparative growth of the ovarian tumour in both sexes one must not overlook the fact that we are dealing with ovarian *auto*grafts in females and with *homo*grafts in males. There is also the conflicting fact that in the work of Klein (1953) the incidence of intrasplenic ovarian tumours was even higher in males than in females!

Quite different is the situation when dealing with strain differences. The strain differences between C57bl and Balb A become fully evident when comparing averages in I/V and III/VII. Indeed, as already insisted upon, the notion of the average "size" of these experimental tumours is a very inexact one on account of the heterogeneous structure of the tumours and on account of the bewildering individual variations. But the existence of strain differences in the growth of the tumour becomes more evident when considering the microscopical structure of the tumours.

Microscopical observations

As known from former work of the authorities the intrasplenic ovarian growth in mice is predominantly a granulosa-cell tumour (Fig. 8, 9, 10, 12). But the growth may contain also luteinized tissue (Fig. 11, 13) or may be a pure luteoma (Fig. 14). We have classified the microscopical results in Table II according to the following rules: pure granulosa-cell tumours were pooled together with mixed tumours in which granulosa-cell nodules predominated; pure luteomata which were rare, were pooled with mixed tumours in which nodules of luteinized cells predominated. In Groups I and III of Table II an overwhelming number of non-cortisone animals of the C57bl strain had granulosa-cell tumours, or tumours in which granulosa-cell nodules predominated; no less than 23 out of 30 tumours

(77 per cent) consisted predominantly of granulosa-cells. On the contrary, in the Balb A strain granulosa-cell nodules predominated in only 9 out of 25 (36 per cent) non-cortisone animals with tumours (V/VII).

The last statement acquires considerable interest in view of the fact that the species difference to which attention has been attracted in former work, that of mice and guinea-pigs, manifests itself in the same evolutional trend. The intrasplenic ovarian tumour in the guinea-pig is, in the course of the first years, luteoma (Lipschutz *et al.*, 1946 ; Iglesias, Mardones and Lipschutz, 1953) : the appearance of the granulosa-cell tumour is a very late phenomenon (Mardones, Iglesias and Lipschutz, 1955). Likewise, as already insisted upon, the incidence of luteomata was considerably greater among the ovarian tumours in strain Balb A growing more slowly than in strain C57bl.

In view of these comparative findings it is very remarkable that the inhibitory action of cortisone which expresses itself in the smaller size of the tumour, is paralleled by a greatly diminished incidence of granulosa-cell tumours. This diminution was fully evident in I/II, III/IV and V/VI, but not in males Balb A VII/VIII in which the incidence of granulosa-cell tumours was already small in the non-cortisone group.

The slowing down of tumour growth manifests itself also in the greater incidence of animals in which there is no tumour at all, or in which disorderly growing lutein tissue does not reach a degree which would allow one to designate the tiny strands or nodules of lutein cells present as growths or tumours (Column 6 of Table II ; Fig. 15 and 16). Thus, there were, among the 50 animals with cortisone, no less than 13 animals, or 26 per cent, in which it would have seemed preposterous, or say too liberal, to speak of luteomata. On the contrary, among the 57 non-cortisone animals there were only 2 grafts, or about 4 per cent, of this type, both grafts belonging to the Balb A groups (V and VII).

In 11 out of 32 cortisone animals of the C57bl strain (II and IV) a pronounced degeneration and hyalinization of the tumoural tissue took place (Fig. 17 ; see also Fig. 13 and 16). In most of these animals the hyalinized cells appeared to have been originally luteinized. There were necrotic masses, sometimes with leucocytic infiltration (Fig. 17) : or the greater part of the growth was replaced by loose and oedematous connective tissue. Only one similar case occurred in the Balb A strain (VIII).

As already insisted upon Table II gives also good evidence of strain differences between C57bl and Balb A ; no less than 16 out of 25 non-cortisone animals of Balb A with tumours (64 per cent ; V and VII) had luteomata whereas luteomata occurred in only 7 out of 30 non-cortisone animals of C57bl with tumours (23 per cent ; I and III). This statement is of great interest as there is seemingly not much difference between various strains as to incidence of tumours in the intrasplenic ovarian graft as shown also by the work of Klein (1952, 1953) with various strains (see also Gardner, 1955). Indeed, one must not overlook that figures referring to incidence of tumours depend on the arbitrary criterion in classifying the disorderly grown ovarian tissues as tumourous or non-tumourous.

Discussion

Full evidence is offered that metastatic spread of intrasplenic ovarian granulosa-cell tumours or luteomata was not stimulated by the quantitites of cortisone acetate we have administered. Indeed, one may insist that the quantities absorbed

from cortisone pellets were smaller than those given in daily injections by the authorities who have worked with transplanted tumours. However, on the other hand, one may argue that in our work absorption was a *continuous* one which greatly enhances the activity of steriods. This was evidenced also in preliminary experiments in which pellets of pure cortisone acetate were implanted subcutaneously, with an absorption of about 400 μg. per day. The animals died, without exception, at about 30 days of cortisone treatment.

In our experiments the action of cortisone acetate was prolonged for 4 to 6 months, without metastases being produced, or better without metastatic spread being enhanced, since in experiments of longer duration metastases occurred in non-cortisone animals of one of the strains used.

On the other hand, the quantities of cortisone acetate given in our experiments which failed to enhance metastatic spread, were sufficient to interfere in a deep-going manner in the evolution of the ovarian tumour. This makes it all the less probable that the failure as to metastatic spread was due to insufficient quantities of cortisone having been administered.

We must now ask in which way cortisone produced inhibition of growth of the intrasplenic ovarian tumour. At the actual moment no definite statements can be made as to this. Retardation of growth of transplantable tumours has been supposed to be due to a change in the environment of the tumour and more specifically to the delay in new blood vessel formation (Antopol, Glaubach and Graff, 1954). In our work the action of cortisone was made to begin only about 4 to 6 months after grafting. There were signs of degeneration in 12 out of 50 animals with cortisone (Table II). This condition can be brought in harmony with the concept of the inhibitory action of cortisone being localized in the immediate environment of the tumour. But certain observations would allow for another explanation which we must discuss here.

One of the outstanding differences between the cortisone and non-cortisone groups was the considerable diminution of the incidence of granulosa-cell tumours which took place in all groups, with the exception of the Balb A males in which the incidence of granulosa-cell tumours was already small in the non-cortisone group. The diminution of incidence of granulosa-cell tumours in C57bl females under the influence of cortisone was paralleled by an increased incidence of animals with luteomata plus animals with luteomatous nodules not yet deserving to be classed as tumourous. Most cases with degeneration also belonged to the luteomatous pattern. The same seems to be true for males C57bl where there was only one animal with luteoma but a large number of animals with degenerated luteomatous tissue or animals with small luteomatous nodules not classed as tumours. Since the evolution of the intrasplenic ovarian growth—granulosa-cell tumours or alternatively luteomata—depends undoubtedly on a specific pattern of the uncontrolled delivery of hypophysial gonadotrophic hormones one may tentatively suggest that the antitumourigenic action of cortisone was due to an interference with the production or delivery of these hormones. The ovarian tumoural constellation in C57bl in which granulosa-cell tumours predominate approaches, under the influence of cortisone, that of the guinea-pig in which luteomata or small luteomatous nodules predominate in the first two years aftet grafting. Column 8 of Table II summarizes the displacement from predominance of the granulosa-cell tumour to predominance of the luteomatous reaction. It may be mentioned here that in our work with the *anti*luteinizing action of different

steroids (Mardones, Iglesias and Lipschutz, 1956) cortisone has been found to be void of any such action (unpublished experiments of Dr. S. Figueroa and others).

All happens as if, in the guinea-pig, a steroid homeostasis different from that in strain C57bl were responsible for the tumoural evolution of the ovarian graft characteristic of this species. Thus it would seem that *the difference as to ovarian tumoural evolution between guinea-pig and strain C57bl can be expressed in terms of a genetically given different pattern of the steroid homeostasis in the mentioned two species.* The same might be true for the smaller differences between strains of mice as those between females of C57bl and Balb A in our Table II. And cortisone is not the only steroid which might be in play.* Evolution of tumours in intrasplenic grafts in mice has been inhibited by oestrogen and androgen (Li and Gardner, 1949): evolution of luteomata in guinea-pigs has been inhibited by progesterone (Iglesias, Lipschutz and Mardones, 1950).

The conclusion drawn in our former paper that the difference between mice and guinea-pigs must be expressed in terms of a differential time of evolution of the respective type of malignant neoplastic growth (Mardones, Iglesias and Lipschutz, 1955) always remains valid. Our suggestion that a genetically given pattern of steroid homeostasis may be in play here, refers only to one of those *mechanisms* by which the differential evolution is effected or interfered with.

The antitumourigenic action of cortisone, if effected via the hypophysis, would offer another aspect of great interest. Interference of cortisone, which is a *corticoid*, in the production or delivery of *gonadotrophic* hormones would be a new and very striking example of the intimate relationship existing between the various organotrophic functions of the hypophysis ; tumours of the *suprarenal* are induced in certain strains of mice or rats by castration, i.e. presumably by a deficiency of *ovarian* hormones. Hitherto the organotrophic faculties of the hypophysis have been considered as the sum of independent gonadotrophic, thyrotrophic, corticotrophic, somatotrophic or other functions. At first glance this concept seems to be corroborated by the fact that each of the various organotrophic functions and so also their tumourigenic deviations are related to certain cell types as shown especially by the outstanding experimental work of Furth (1954) and his colleagues on thyrotrophin- or corticotrophin-secreting pituitary tumours. But production of suprarenal tumours by castration as known from classic work of Woolley and others, and the possibility of controlling the tumourigenic deviation of the gonadotrophic function of the hypophysis by cortisone—should our tentative suggestion be right that cortisone has acted not on the tumour itself—show that the organotrophic hypophysial potency is an *integrated whole* and that different organotrophic functions of the hypophysis are *intertwined* in the narrow space of the anterior lobe (see also Section 11 in Lipschutz, 1956).

SUMMARY

Cortisone acetate was administered to castrated mice of two different strains (C57bl and Balb A) bearing intrasplenic ovarian grafts, to study the question whether metastatic spread of a tumour growing under the given experimental conditions could be enhanced by this steroid. Cortisone was allowed to act 4 to 6 months through continuous absorption from subcutaneously implanted pellets.

Metastatic spread was not enhanced in the cortisone animals.

* Neither is cortisone an antitumourigenic steroid *per se* ! Survival and proliferation of human tumours transplanted into rats is rendered possible by the administration of cortisone (Toolan, 1953).

The quantities of cortisone acetate administered greatly slowed down the growth of the intrasplenic ovarian tumours in both strains used.

The microscopical structure of the tumours also was fundamentally influenced by cortisone : the incidence of granulosa-cell tumours greatly diminished and the incidence of luteomata plus luteomatous nodules increased.

The small luteomatous ovarian growths, tumoural or non-tumoural, in the two strains of mice treated with cortisone were similar to the slowly growing intrasplenic ovarian growths in the guinea-pig in the first two years after grafting.

The tentative suggestion is made that the antitumourigenic action of cortisone was by interference with the production or delivery of gonadotrophic hypophysial hormones on which ovarian tumourigenesis in intrasplenic grafts depends.

If this interpretation were true the results obtained would suggest that differences as to the evolution of intrasplenic ovarian tumours between guinea-pigs and mice, or between different strains of mice, should be explained by a genetically determined difference in the pattern of the steroid homeostasis.

The results obtained would likewise offer a new example of how a tumourigenic deviation of the *gonadotrophic* function of the hypophysis can be influenced by a corticoid whose production in the body is dependent on the *corticotrophic* function of the hypophysis.

Cordial thanks are due especially to Miss Socorro Salinas for generous help at operations and necropsies, and to Mr. Alejandro Castillo, photographer.

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FURTHER EXPERIMENTAL OBSERVATIONS ON THE LATE EFFECTS OF THOROTRAST ADMINISTRATION

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In a recently published paper (Guimaraes, Lamerton and Christensen, 1955) pronounced pathological changes and increased incidence of neoplasms in mice injected intravenously with thorotrast were reported. A detailed description was given of the cytological and histological changes observed in the liver, lungs and spleen. These findings were comparable in many ways with those of Johansen (1955) who studied the late effects of thorotrast injection in rabbits, particularly with regard to the malignant reticulo-endotheliomas observed in both species.

The work reported in the present paper comprises a histological study of the tissues of thorotrast-injected and control mice sacrificed at regular intervals, in an endeavour to learn something of the sequence of changes which culminate in the production of neoplasms, particularly those observed in the liver.

EXPERIMENTAL

Plan of Experiment

Fifty Schofield mice were injected intravenously with 0·1 ml. thorotrast, of a commercial preparation of recent manufacture, and divided into groups of five animals. Four mice died soon after the injections and six more died at different intervals during the experiment. With the exception of one mouse which died nine months after the injection, none that died prior to sacrifice showed any gross lesion. Groups of animals were killed every month after the injection up to the sixth month. After that time the animals were sacrificed every two months. In the first five months five animals were killed on each occasion, in the sixth and eighth months four animals, and in the tenth and twelfth months three and four animals respectively. In all cases the same number of controls were sacrificed simultaneously.

Sections were taken from liver, spleen, lungs, kidneys, adrenals and bone marrow. The stains used were haematoxylin-eosin, Maximov and Feulgen with phosphotungstic acid haematoxylin for mitochondria, and Gomori's stain for acid phosphatase.

Histological Findings

The Liver

In the liver the types of changes observed in the thorotrast-injected animals were those described in detail in the earlier paper. The giant and distorted nuclei with the spherical " inclusion " bodies were first observed in the thorotrast animals four months after injection, and were seen fairly regularly in all injected

mice after that time. The intensity of the changes was, however, extremely variable, being marked in some animals and just discernible in a few others, having no apparent relationship with the amount of thorotrast present but showing definite increase with time. Reticulo-endothelial proliferation, of the type shown in Fig. 1, was significant from the sixth month onwards, but here again in a few specimens was almost entirely negligible. Two injected animals showed malignant reticulo-endotheliomas (Fig. 2(a), 2(b), 3(a) and 3(b). One of these animals died at nine months and owing to post-mortem changes only the liver could be examined histologically. The increased size of the spleen, however, suggested that the tumour was already disseminated. The second reticulo-endothelioma was found in the animal sacrificed twelve months after injection and was still confined to the liver. Hepatomas were observed in five injected animals, sacrificed at six months, eight months, ten months and twelve months (2) after injection. In three of these animals the hepatomas were multiple.

Among the control animals two showed solitary hepatomas, at seven and twelve months respectively, but no generalised liver changes were seen until twelve months, when there were some cell changes and reticulo-endothelial proliferation, rather pronounced in two specimens.

MacCardle and Congdon (1955) have recently claimed to have observed changes in the mitochondria of hepatic cells of mice given 500 to 1200r of X-radiation. We were unable to detect any change in the morphology or arrangement of the mitochondria in the thorotrast-injected mice (Fig. 4 and 5). Depending on their position in the lobule, the cells both of injected and control animals showed the same pattern of globulated, filamentous and rod-like mitochondria. Some fragmentation and vesiculation of the mitochondria were observed in both treated and control animals. These appearances are probably related to metabolic and ageing processes.

EXPLANATION OF PLATES.

FIG. 1.—Pre-malignant reticulo-endothelioma proliferation in the liver (mouse killed at twelve months after thorotrast injection) H. & E. × 80.

FIG. 2(*a*).—First stage of malignant reticulo-endothelioma, still confined to the liver. Liver cell changes present (Mouse killed at twelve months after thorotrast injection.) H & E. × 80.

FIG. 2(*b*).—Same animal as Fig. 2(*a*). Another part of the liver, showing disruption of the sinusoid wall and of the trabecular arrangement. Atrophy of liver cells. H. & E. × 80.

FIG. 3(*a*).—More advanced stage of malignant reticulo-endotheliomas (Mouse died at nine months.) H. & E. × 80.

FIG. 3(*b*).—Same animal as Fig. 3(*a*), showing overgrowth of the liver parenchyma by the tumour cells. H. & E. × 80.

FIG. 4.—Mitochondria in liver of normal animal. Stain-phosphotungstic acid haematoxylin. × 500.

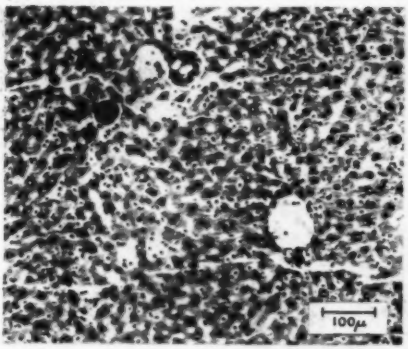
FIG. 5.—Mitochondria in liver of thorotrast-injected animal (mouse killed at twelve months after injection). × 500.

FIG. 6.—Acid phosphatase distribution in liver of normal animal. Gomori's stain. × 360.

FIG. 7.—Acid phosphatase distribution in liver of thorotrast-injected animal. The large black area is a deposit of thorotrast (Mouse killed at twelve months after injection.) Gomari's stain. × 360.

FIG. 9.—Thorotrast aggregates in liver of animal killed at 2 months. H. & E. × 85.

FIG. 10.—Thorotrast aggregates in liver of animal killed at 12 months. H.& E. × 85.



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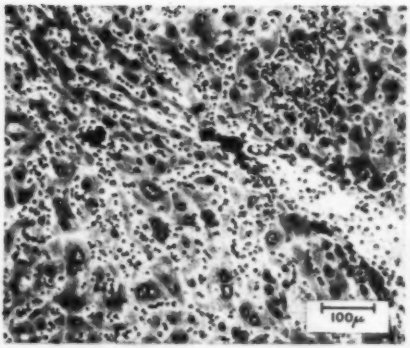

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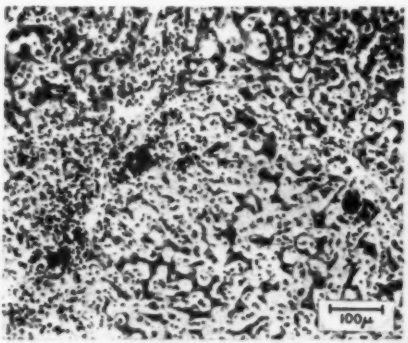

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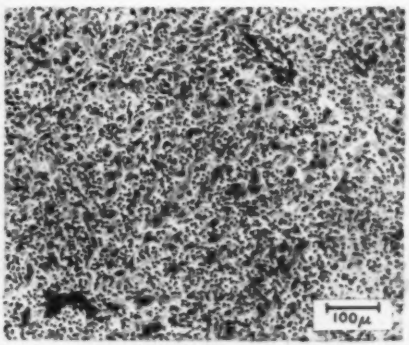

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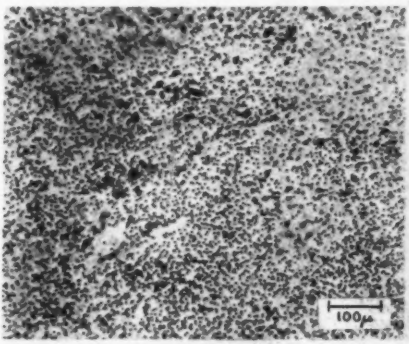

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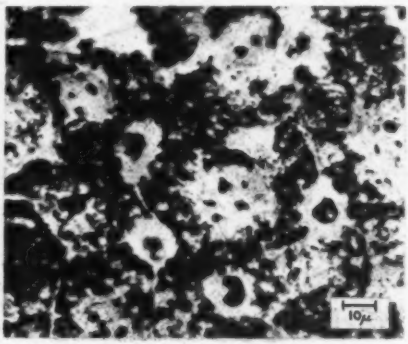

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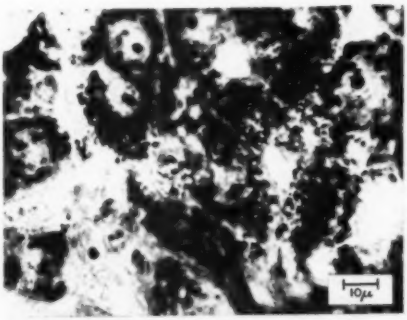

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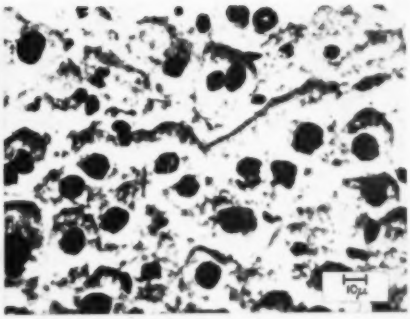

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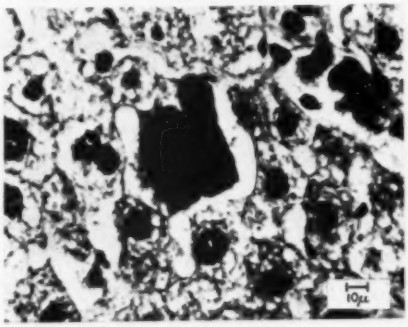

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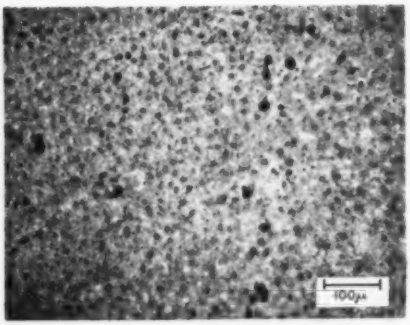

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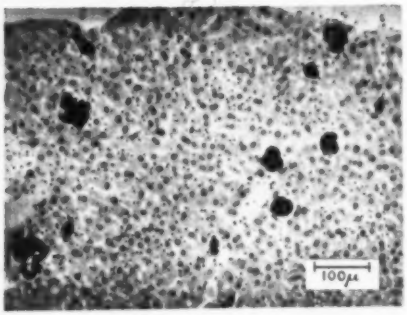

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The hepatomas found in both injected and control animals show the usual increased basophilia in the cytoplasm. The amount of basophilic substance in the hepatic cells appeared not to be affected by the presence of thorotrast.

The distribution of acid phosphatase in the liver followed the same pattern both in control and injected animals (Fig. 6 and 7). Some degree of fatty degeneration was found in both groups of animals.

In order to study the mitotic activity of the tissues the two groups of animals sacrificed at twelve months were given 0·1 mg. of colchicine six hours before death. No differences were observed between injected and control animals, apart from the fact that the mitotic index within the hepatomas in the two injected animals was about twice that within the hepatoma in the control animal.

In none of the animals, injected or control, was connective tissue proliferation or a picture of cirrhosis seen at any time.

Other tissues

In the lung the amount of thorotrast seen was very small compared with liver or spleen, and none of the large deposits observed in the previous experiment were seen. Multiple lung adenomas of the type described in the previous paper were found in eight of the injected animals, sacrificed at five months, six months (2), eight months, ten months (2) and twelve months (2). In the control animals four lung adenomas of the same type were found in animals sacrificed at six months, ten months and twelve months (2).

The concentration of thorotrast seen in the spleen was comparable with that in the liver. No changes were observed in this experiment in the spleen apart from increased cellularity of the red pulp in a few specimens.

A very small amount of thorotrast was found in kidney and bone marrow, but no damage was observed in these or other tissues examined.

Radiation dosage

The estimation of the radiation dosage received by any tissue containing thorotrast aggregates is a formidable problem. The dose received will depend on :

(*a*) The "age" of the thorotrast, that is, the extent to which pure thorium 232 is accompanied by its disintegration products, particularly radiothorium (Th^{228}). This problem has been considered by Rundo (1955).

(*b*) The size of the thorotrast aggregates, which will determine the extent of self-absorption of the emitted alpha particles. The size and number of aggregates will determine the degree of non-uniformity of radiation dose throughout the tissue.

(*c*) The movement of thorotrast particles within the tissue.

Rotblat and Ward (1953) have taken account of factors(*a*) and (*b*) in their autoradiographic studies of thorotrast aggregates in tissue specimens from patients who had received thorotrast intravenously some years previously. On the basis of the terminal distribution of the thorotrast aggregates they find (personal communication) a mean value for the radiation dose in the liver of about 2 rep/week for an intravenous injection of 20 ml. of thorotrast. The value of 2 rep/week represents an average over the whole organ. In the immediate neighbourhood of the aggregate the dose may be many times this value.

In the present mouse experiments it has not been possible to carry out detailed autoradiographic studies of the type made by Rotblat and Ward (1953), but if

one were to assume that the liver of the mouse took up the same fraction of the injected material and that the size, distribution and "age" of the thorotrast aggregates were the same as in the human case the mean dose to the liver would be of the order of 20 rep/week since, on a body weight basis, the mice received ten times as much as in the human case. This value can only be taken as giving an order of magnitude for the terminal dosage rate since in the mouse experiments the thorotrast used was of recent manufacture and at 12 months (when the last group of mice was killed) the average size of the thorotrast aggregates was probably considerably greater than in the human cases studied by Rotblat and Ward (1953).

A study of the growth in size of the aggregates with time has been made in the present experiments and the results are plotted in Fig. 8 which shows the

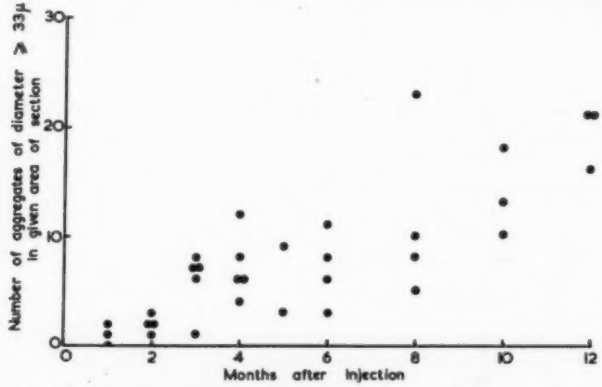

FIG. 8.—Number of thorotrast aggregates above 33μ mean linear dimensions in given area (4·7 mm.^{2}) of 5μ liver sections at various times after injection.

number of aggregates of mean linear dimensions greater than 33μ in a given area (4·7 mm^2) of a 5μ section of liver, for sections taken at 1, 2 . . . 12 months after injection. Each point represents counts on a separate slide and, although there is considerable scatter, the increase with time in the number of large aggregates is very evident. Thus there was a considerable movement of thorotrast within the mouse liver tissue, at least up to 12 months after injection. The consequence of this is that the liver tissue will receive a much more uniform radiation dose than would be expected on the basis only of the terminal distribution of the aggregates, which may explain why the cytological changes observed in the sections are not seen only in the neighbourhood of the aggregates.

Typical areas of sections at 2 and 12 months respectively are shown in Fig. 9 and 10.

DISCUSSION

In the earlier experiment (Guimaraes, Lamerton and Christensen, 1955) histological studies were made only on thorotrast-injected mice, which died or were sacrificed thirteen to twenty-one months after injection. In these animals one malignant reticulo-endothelioma in the liver and five hepatomas were found, at

least one malignant haemangio-endothelioma in the spleen and seven lung adenomas. The present study, extending over a shorter time (maximum of twelve months) showed clearly that the incidence of neoplasms is greater in injected than in control animals. The malignant neoplasia observed, two reticulo-endotheliomas, both occurred in injected animals. Of the non-malignant neoplasia five hepatomas and eight lung adenomas were found in the injected animals, while in the control animals two hepatomas and four lung adenomas were found. No malignant haemangio-endotheliomas in the spleen were observed in the present series, but this may have been because of the shorter duration of the experiment, since in the previous study the only certain tumour of this type was found in the animal sacrificed twenty months after injection.

The present study has given no indication that tissue destruction of any sort precedes the development of the liver neoplasia even although the radiation dosage is very large. The development of the malignant reticulo-endotheliomas can be traced back to a directly induced reticulo-endothelial proliferation, as shown in Fig. 1, 2(a), 2(b), 3(a) and 3(b). This proliferation leads to a disruption of the sinusoid wall, with oedema of the Disse spaces and blood extravasation later. Liver cells in this area show atrophy and regressive changes. The reticulo-endothelioma overgrowth then steadily and progressively replaces the liver parenchyma. Although the mouse reticulo-endotheliomas appear to belong to the same basic cellular type as those described by Johansen (1955) in his thorotrast treated rabbits, they do not show vascular formation and they apparently arise in the liver, disseminating in later stages, whereas in the rabbits they appear to be of a multifocal origin.

In the case of the hepatomas in the injected mice, not only was there an absence of preceding tissue destruction, but there was no apparent relationship between the intensity of the degenerative cell changes and the incidence of the hepatomas.

The alveolar cell proliferation in the lung preceding tumour formation was again seen in the present series, but the granulomatous reaction reported by Johansen (1955) in rabbits was not observed.

A number of lines of experimental work have suggested that the carcinogenic effect of radiation can be interpreted as a form of reaction to gross tissue damage and destruction produced by the radiation. There was, however, no evidence of gross tissue damage in the present study. It may be that certain of the changes produced by thorotrast can be considered as a speeding up of natural processes, and it is of interest that some cell changes and moderate reticulo-endothelial proliferation were seen in two of the control animals killed at twelve months. It is not known to what extent malignant reticulo-endothelioma will appear spontaneously in this strain of mice at a later stage, and this is intended as the subject of a future study.

The carcinogenic effect of thorotrast may, of course, be related to some extent to the physical presence of the particulate material in the tissue or even to chemical properties of thorium. These are aspects of the problem which so far have received no serious study, but must be investigated before any mechanism of the action of thorotrast can be put forward with confidence.

The present study gives clear evidence of the increase in average size of thorotrast aggregates from 1 month up to at least 12 months, when the last group of mice was sacrificed.

SUMMARY

In continuation of a previous investigation (Guimaraes, Lamerton and Christensen, 1955) a histological study has been made of the tissues of thorotrast injected and control mice sacrificed at intervals up to one year. It appears that the development of reticulo-endotheliomas of the liver is not the consequence of any gross tissue destruction and repair, but follows a generalised reticulo-endothelial proliferation. In the case of the hepatomas appearing in the injected mice there was also no evidence of preceding tissue destruction.

This study has also given evidence of the increase with time in the mean size of thorotrast aggregates in liver tissue.

It is a pleasure to acknowledge our indebtedness to Miss Adams, Mr. Gibbs, Miss Gwyther and Miss Winsborough for their valuable help, and to Professor D.W. Smithers, Director of the Radiotherapy Department and Professor W. V. Mayneord, Director of the Physics Department of the Institute of Cancer Research.

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THE CARCINOGENIC ACTIVITY OF 2-NAPHTHYLAMINE

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HUEPER (1938) and Bonser (1943) established that carcinoma of the bladder may be induced by feeding 2-naphthylamine to dogs over a protracted period. In the more recent experiments of Bonser, Clayson, Jull and Pyrah (1952) the oral administration of 2-naphthylamine to mice resulted in a significant yield of liver tumours, but in 5 rabbits which survived 4 years of treatment the only changes induced were a small papilloma of the bladder in one and advanced epithelial hyperplasia in another. Bonser, Clayson and Jull (1951) presented evidence that 2-naphthylamine is carcinogenic by virtue of its conversion in the body to 2-amino-1-naphthol. On the basis of these findings, a hypothesis covering the mode of action of the aromatic amines in general has been put forward (Clayson, 1953). However, doubts as to the validity of these postulates have persisted due in part to the fact that in a few cases the injection of oily solutions of 2-naphthylamine (Hackmann, 1951) or of extracts of the chemical (Case and Pearson, 1952) has resulted in the appearance of subcutaneous sarcomas. Thus there was the possibility that the carcinogenic activity of 2-naphthylamine was due to an impurity present to a small extent in the substance used by Bonser (1943) and to a greater extent in the crude industrial material used by Hueper (1938).

The following experiments were carried out to test the local and distant carcinogenic properties of 2-naphthylamine following its subcutaneous injection into mice, and to discover whether the chemical, highly purified by gradient sublimation, as developed by Dr. R. A. M. Case (*cf* Henson, Somerville, Farquharson and Goldblatt, 1954), was carcinogenic to dogs.

MATERIALS

2-*Naphthylamine* purified as described by Bonser (1943) and supplied by British Drug Houses Ltd., was termed BDH.

2-*Naphthylamine* purified by the process of gradient sublimation described by Henson *et al.* (1954) was stored before use at 0° C. and was termed RCH.

Mongrel dogs were used.

All the organs were examined *post mortem* and the bladder, liver and kidneys were examined microscopically. The bladder of one dog (86) was examined by cystoscope during life.

For experiments 2–4, Swiss type albino mice, approximately 12 weeks of age, were obtained in one batch from a dealer. Some spontaneous tumours of lymphoid tissue and one ovarian and one breast tumour occurred in a control group injected with arachis oil only but no hepatomas were observed among 11 mice surviving for 70 weeks or more.

The CBA mice were from a colony inbred for over 70 generations. Bonser *et al.* (1952) reported an incidence of 8·1 per cent of hepatomas in breeding mice of this strain. During the period of the present investigation one hepatoma was observed in a female at 123 weeks of age among 5 male and 10 female breeding mice which survived more than 78 weeks. The mice were approximately 12 weeks of age at the commencement of treatment.

The livers of all the mice, both control and experimental, were examined microscopically. In all but a very few mice living for 50 weeks or more after the beginning of treatment there were small collections of inflammatory cells (polymorphs, lymphocytes and fibroblasts) in the portal tracts. In the livers of experimental mice in which hepatomas occurred, there was no greater degree of cellular infiltration of the portal tracts. There was, however, mild proliferation of the bile ducts in many of the livers and in a few there were small benign cholangiomatous areas.

Experiment 1

2-Naphthylamine (RCH) was given orally in a gelatine capsule 6 days a week to 4 female dogs. Initially the dose was 200 mg. but this was increased after 6 months to 600 mg. The maximum cumulative dose of chemical administered to a dog was 310 g.

Dog 87 (female) died after 14½ months of treatment with no neoplastic changes in the bladder.

Dog 95 (female) received 2-naphthylamine for 1 year only and was killed 1¾ years after the beginning of treatment. There were no neoplastic changes in the bladder epithelium.

Dog 89 (female) died after 2 years of treatment. There were over 40 transitional cell papillomas up to 2 cm. in diameter in the bladder (Fig. 1). Although some were regarded as histologically malignant none was invasive.

Dog 86 (female). On cystoscopic examination of the bladder 2 years after the beginning of treatment epithelial elevations and congestion were seen. Feeding of the chemical was then stopped. On re-examination ten months later two papillary tumours were seen, one of which was clinically malignant. This dog was killed 3 years after treatment had been started. At *post mortem* examination, many papillomas were present in the bladder, the picture being comparable with that in Dog 89. The tumours were of transitional-cell type. One was a frank carcinoma and was invading the muscle of the bladder wall.

Experiment 2

A 3 per cent solution of 2-naphthylamine (BDH) in arachis oil was allowed to stand for at least 4 weeks. 0·1 ml. of this solution was injected subcutaneously into albino mice twice weekly for 50 weeks.

Ten of 16 mice surviving 20 or more weeks of treatment developed subcutaneous sarcomas near the injection sites (Table I). Four of 5 mice dying after 80 weeks had hepatomas and in three there were areas of cholangioma (Table II).

Experiment 3

0·1 ml. of a 3 per cent solution of 2-naphthylamine (BDH) in arachis oil, prepared immediately before use, was injected subcutaneously into albino mice

TABLE I.—*Incidence of Subcutaneous Sarcomas in Experiments* 2–6

Weeks of treatment

TABLE I.—*Incidence of Subcutaneous Sarcomas in Experiments 2–6*

Experiment No.	Type of mouse.	Injected material.	Sex.	Weeks of treatment. 21–30.	31–40.	41–50.	51–60.	61–70.	71–80.	Over 80.	Total tumour incidence.	Percentage tumour incidence.
2	Albino	BDH 2-naphthylamine left to stand in oil	M	2/2	1/1	1/2	—	—	—	0/2	4/7	10/16	63
			F	1/1	2/2	1/2	1/1	—	—	1/3	6/9	
3	Albino	BDH 2-naphthylamine freshly prepared in oil	M	—	0/3	0/2	0/1	0/2	—	—	0/8	0/13	0
			F	—	—	—	—	0/1	0/1	0/3	0/5	
4	Albino	RCH 2-naphthylamine freshly prepared in oil	M	—	—	1/3	0/2	0/2	—	—	1/7	2/12	17
			F	—	1/1	0/1	—	0/3	—	—	1/5	
5	CBA	RCH 2-naphthylamine HCl. freshly prepared in water	M	—	—	—	0/1	0/1	0/2	0/5	0/9	0/10	0
			F	—	—	—	—	—	—	0/1	0/1	
6	CBA	BDH 2-naphthylamine HCl. freshly prepared in water	M	—	—	—	0/1	—	—	0/8	0/9	0/11	0
			F	—	—	—	—	—	—	0/2	0/2	
Control albino mice		Arachis oil only	M	—	—	—	0/1	—	0/4	0/3	0/8	0/17	0
			F	—	—	0/1	—	0/4	0/1	0/3	0/9	

Numerator denotes number of mice with tumours dying within the stated period.
Denominator denotes number of mice dying within the stated period.

TABLE II.—*Incidence of Hepatomas in Experiments 2–6*

Experiment No.	Type of mouse.	Injected material.	Sex.	Weeks of treatment. 21–30.	31–40.	41–50.	51–60.	61–70.	71–80.	Over 80.	Total tumour incidence.	Percentage tumour incidence.
2	Albino	2-naphthylamine BDH left to stand in oil	M	0/2	0/1	0/2	—	—	—	2/2	2/7	4/16	25
			F	0/1	0/2	0/2	0/1	—	—	2/3	2/9	
3	Albino	BDH 2-naphthylamine freshly prepared in oil	M	—	0/3	0/2	0/1	0/2	—	—	0/8	2/13	15
			F	—	—	—	—	0/1	1/1	1/3	2/5	
4	Albino	RCH 2-naphthylamine freshly prepared in oil	M	—	—	0/3	1/2	0/2	—	—	1/7	1/12	8
			F	—	0/1	0/1	—	0/3	—	—	0/5	
5	CBA	RCH 2-naphthylamine HCl. freshly prepared in water	M	—	—	—	0/1	0/1	1/2	1/5	2/9	2/10	20
			F	—	—	—	—	—	—	0/1	0/1	
6	CBA	BDH 2-naphthylamine HCl. freshly prepared in water	M	—	—	—	0/1	—	—	4/8	4/9	4/11	36
			F	—	—	—	—	—	—	0/2	0/2	
Control albino mice		Arachis oil only	M	—	—	—	0/1	—	0/4	0/3	0/8	0/17	0
			F	—	—	0/1	—	0/4	0/1	0/3	0/9	
Control CBA mice		Breeding only	M	—	—	—	—	—	—	0/5	0/5	1/15	7
			F	—	—	—	—	—	0/1	1/9	1/10	

Numerator denotes number of mice with tumours dying within stated period.
Denominator denotes number of mice dying within the stated period.

twice weekly for 50 weeks. None of 13 mice which survived 33 weeks or more of treatment developed sarcomas, but 2 of 4 mice living over 77 weeks had hepatomas (Tables I and II).

Experiment 4

The treatment was similar to that in Experiment 3, except that a freshly prepared solution of 2-naphthylamine (RCH) was used.

Local sarcomas occurred at 37 and 41 weeks of treatment in 2 of 12 mice surviving for 37–69 weeks. One cholangioma associated with cirrhosis was observed at 43 weeks and one small hepatoma at 52 weeks. The last mouse in this group died at 69 weeks, 8 weeks before the first hepatoma was seen in Experiments 2 or 3.

Experiment 5

0·1 ml. of a 3 per cent solution of 2-naphthylamine hydrochloride (RCH), freshly prepared in warm water, was injected subcutaneously into CBA mice twice a week for 6 months and then once a week for a further 4 months.

No sarcomas were observed in any of 10 mice surviving more than 58 weeks. Hepatomas were seen at 77 and 93 weeks in 2 of 8 mice dying after 70 weeks (Table II).

Experiment 6

The treatment was the same as in Experiment 5, except that 2-naphthylamine hydrochloride (BDH) was used.

No sarcomas occurred in any of the 11 mice surviving more than 56 weeks of treatment. Hepatomas were seen in 4 of 11 mice dying after 82 weeks.

DISCUSSION

The induction of bladder tumours in the two dogs of Experiment 1 which lived for two years or more is evidence that 2-naphthylamine purified by the most stringent means is as potent a bladder carcinogen as the chemical used by Bonser (1943). A summary of the results of treatment with 2-naphthylamine in all the dogs investigated in Leeds since 1938 is given in Table III. Every dog treated for 2 years or longer has developed bladder tumours. Few other tests of carcinogens have yielded tumours in all the experimental animals. These facts would support the view that the carcinogen is concentrated in the urine. As yet there has been no report that tumours occur in other sites in dogs fed with 2-naphthylamine.

The experiments with mice, designed to test whether 2-naphthylamine is a local or distant carcinogen, or both, have shown that the incidence of local sarcomas can be greatly reduced by using freshly prepared solutions in oil, or by avoiding oily solutions altogether and using aqueous solutions of the hydrochloride. In

EXPLANATION OF PLATE.

FIG. 1.—Dog 89, female, oral 2-naphthylamine RCH for 2 years. Bisected bladder showing multiple papillomata, ranging in size from pinhead to 2 cm. diameter. Most of the tumours are slightly pedunculated, but the largest one (not seen here) was sessile, with a hard base. × 1·5.

Bonser, Clayson, Jull and Pyrah.

TABLE III.—*Dogs treated with 2-naphthylamine by mouth*

Dog No.	Sex.	Survival (years).	Source of chemical.	Bladder tumours.	Other observations.
I	M	1	BDH	−*	
87	F	1¼	RCH	−	
2	F	1½	BDH	−	
95	F	1¾	RCH	−	Received chemical for one year only
89	F	2	„	+	
86	F	3	„	+	Received chemical for 2 years only
II	F	3⅔	BDH	+*	
III	M	4	„	+*	
1	F	4¼	„	+	
3	M	4½	„	+	
IV	M	5	„	+*	
5	F	5	„	+	Sodium bicarbonate daily
6	F	5¼	„	+	„ „ „
7	M	5¼	„	+	„ „ „
4	F	5¾	„	+	

* Described by Bonser, 1943.

Experiment 2, in which 2-naphthylamine (BDH) in oily solution was injected after standing for several weeks, an incidence of 63 per cent of local sarcomas was obtained (Table I), but in Experiments 3 and 4 in which freshly prepared oily solutions of 2-naphthylamine (BDH and RCH) were used, the incidence of these tumours was reduced to 8 per cent. In Experiments 5 and 6, in which the aqueous hydrochloride was used, local sarcomas were eliminated altogether. It is deduced from these results that on standing oily solutions of 2-naphthylamine develop local carcinogenic properties. A similar change may well take place when freshly prepared oily solutions are introduced into the subcutaneous tissues, thus accounting for the small yield of sarcomas in mice which received such solutions.

In assessing the significance of the occurrence of hepatomas, it should be noted that these arose late, i.e. with one exception after the 70th week. From Experiments 5 and 6 it is apparent that hepatomas can be induced by 2-naphthylamine hydrochloride without accompanying sarcomas. The induction time of liver tumours in all experiments was, with one exception, between 77 and 91 weeks, but only one sarcoma occurred after 54 weeks. It is thus possible that the causative agent of the sarcomas in these experiments is different from that which induced the hepatomas. The hepatoma incidence in mice treated with 2-naphthylamine was 66 per cent after 70 weeks or more, and with 2-naphthylamine hydrochloride 33 per cent. These results are comparable with those reported by Bonser *et al.* (1952), within the limits of the small number of effective mice.

SUMMARY

1. Oral administration of very pure 2-naphthylamine induced multiple tumours of the bladder in 2 dogs surviving 2 years or more of treatment.

2. Local sarcomas were induced in 63 per cent of mice injected subcutaneously with an oily solution of 2-naphthylamine which had stood for 4 weeks.

3. When freshly prepared oily solutions of 2-naphthylamine were similarly injected the incidence of local sarcomas was reduced to 8 per cent.

4. Subcutaneous injection of 2-naphthylamine hydrochloride did not induce local sarcomas.

5. Although spontaneous hepatomas occurred in control CBA breeding mice a greater incidence occurred in mice which received either specially purified or " partially " purified 2-naphthylamine in the form of injections in oil or as aqueous solutions of the hydrochloride.

6. It is concluded that 2-naphthylamine purified by gradient sublimation is carcinogenic to the dog and the mouse.

The 2-naphthylamine, purified by gradient sublimation, was a gift from the Chester Beatty (K. lab) through the kind offices of Professor F. Bergel, to whom the authors wish to express their thanks.

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A FURTHER STUDY OF THE CARCINOGENIC PROPERTIES OF ORTHO HYDROXY-AMINES AND RELATED COMPOUNDS BY BLADDER IMPLANTATION IN THE MOUSE

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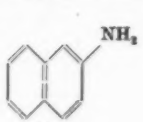
In 1952 Bonser, Clayson, Jull and Pyrah examined the carcinogenic action of 2-naphthylamine (I) and the hydrochloride of one of its metabolites, namely, 2-amino-1-naphthol (II), by means of the implantation of paraffin wax pellets containing the chemicals into the lumen of the mouse bladder. On the basis of experiments on forty-two mice they concluded that whereas the parent amine, 2-naphthylamine, was not active under these conditions the metabolite, 2-amino-1-naphthol was carcinogenic.

The carcinogenicity of 2-amino-1-naphthol and the absence of such activity with 2-naphthylamine led Clayson (1953) to suggest that aromatic amines and certain other compounds were carcinogenic because of their conversion in the animal body to ortho hydroxy-amines. As a first step in testing this hypothesis it was necessary to show that ortho hydroxy-amines are in fact carcinogenic.

It has recently been shown that 4-aminodiphenyl (*para*-xenylamine, VII) elicits biological responses similar to 2-naphthylamine. It is an industrial bladder



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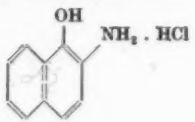

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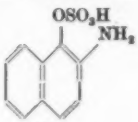

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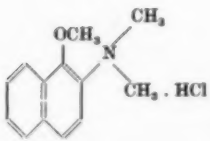

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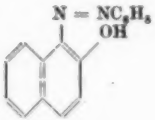

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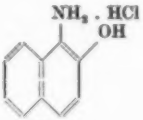
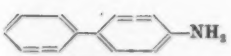
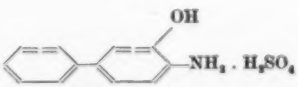


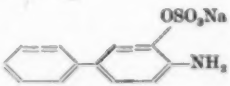
(VI)



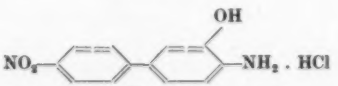
(VII)



(VIII)



(IX)



(X)

carcinogen (Melick, Escue, Naryka, Mezera and Wheeler, 1955): it rapidly induces tumours in the dog (Walpole, Williams and Roberts, 1954: Deichmann, Coplan, Woods, Anderson, Heslin and Radomski, 1956): and it is relatively slow acting in the rat (Walpole, Williams and Roberts, 1952). In the dog 4-aminodiphenyl is excreted into the urine as 4-amino-3-diphenylyl hydrogen sulphate (IX), (Bradshaw and Clayson, 1955), and Bradshaw (unpublished observation) has shown that free 3-hydroxy-4-aminodiphenyl (VIII) is present in low concentration in the urine of dogs receiving the chemical. Kirby and Peacock (1949) found that the erstwhile food colorant 1-phenylazo-2-naphthol (Oil orange E) (V) induced hepatomas in 7 of 24 mice surviving to a significant age and it was suggested (Clayson, 1953) that the activity of this compound might be due to its reduction in the body to 1-amino-2-naphthol (VI).

The purpose of the present experiments was to confirm the results previously obtained with 2-amino-1-naphthol and 2-naphthylamine and to see if they can be extended to 4-aminodiphenyl and 3-hydroxy-4-aminodiphenyl, and to 1-phenylazo-2-naphthol and 1-amino-2-naphthol.

METHODS

Albino mice were used. They were obtained either from a dealer or were from the WLL strain bred in the laboratory.

Pellets of the substance to be tested were prepared from suspensions in paraffin wax (m.p. 56° C.) and were implanted as described by Jull (1951). The results were interpreted by the criteria discussed by Bonser and Jull (1956).

2-*Naphthylamine* was purified either as described by Bonser (1943) or by the method of gradient sublimation under high vacuum as developed by Dr. R. A. M. Case (*cf* Henson, Somerville, Farquharson and Goldblatt, 1954). The samples were termed B.D.H. and R.C.H. respectively.

2-*Amino*-1-*naphthol hydrochloride* was prepared by (i) $Na_2S_2O_4$ reduction of 2-nitroso-1-naphthol (British Drug Houses) in alkaline solution (c.f. Grandmougin, 1906); (ii) by $SnCl_2$ reduction of 2-nitroso-1-naphthol in acid solution, tin salts being removed with H_2S (Grandmougin and Michel, 1892); or (iii) hydrolysis of 2-amino-1-naphthyl hydrogen sulphate with dilute hydrochloric acid (Wiley, 1938). The products obtained by these methods will be referred to as A, B and C respectively. In each case the product was purified by recrystallisation from dilute HCl and had the same absorption spectra in the range 230–360 mμ.

2-*Amino*-1-*naphthyl hydrogen sulphate* was extracted from the urine of dogs receiving oral 2-naphthylamine. The ultraviolet absorption spectra of the chemical obtained in this manner was identical with that obtained by the method of Boyland, Manson and Sims (1953).

2-*Dimethylamino*-1-*methoxynaphthalene hydrochloride* was prepared from 2-nitro-1-methoxy naphthalene as follows; the nitro compound was reduced with H_2 and Raney-Nickel to the amine, which was exhaustively methylated with methyl iodide and Na_2CO_3. 2-(1-Methoxynaphthyl) trimethylammonium iodide was hydrolysed by refluxing with alkali to 2-dimethylamino-1-methoxynaphthalene, which was converted to its hydrochloride.

1-*Phenylazo*-2-*naphthol* was purified chromatographically by the British Drug Houses.

1-*Amino*-2-*naphthol hydrochloride* was prepared by $SnCl_2$ reduction of 1-nitroso-2-naphthol (British Drug Houses) (Groves, 1884) and tin salts were removed by H_2S and the product purified by the method of Fieser (1943).

The *tin complexes* of 2-amino-1-naphthol and 1-amino-2-naphthol were prepared by recrystallising the hydroxyamines from 5 per cent $SnCl_2$ in diluted HCl.

4-*Aminodiphenyl* was obtained from British Drug Houses.

3-*Hydroxy*-4-*aminodiphenyl sulphate* and 4-*amino*-3-*diphenylyl sodium sulphate* were prepared by the method of Boyland and Sims (1954).

4′-*Nitro*-4-*amino*-3-*hydroxydiphenyl hydrogen chloride* was prepared by the method of Bradshaw and Clayson (1955).

RESULTS

In the course of these experiments it became evident that with the compounds under investigation tumours of the bladder epithelium seldom arose before twenty-five weeks. Only mice which survived into this period have been included in the analysis. 605 mice survived longer than 25 weeks and of these 45 (7·4 per cent) were killed on account of failing health between 25 and 30 weeks, 38 (6·3 per cent) between 31 and 35 weeks, 44 (7·3 per cent) between 36 and 40 weeks, while 478 (79 per cent) were killed at 40 weeks. As is shown in Table I, there were no marked differences between the survival times in the various groups.

TABLE I.—*Survival of Implanted Mice Killed After 25 Weeks.*

Experiment.	Compound.	Number of mice killed (weeks). 25–30.	31–34.	35–39.	40.	Total.
1	Paraffin wax alone	8	5	6	37	56
2	2-naphthylamine (I)	8	11	8	62	89
3	2-amino-1-naphthol HCl (II)	5	10	5	98	118
4	„ „ „ + tin	10	1	10	47	68
5	2-amino-1-naphthyl hydrogen sulphate (III)	2	2	2	32	38
6	2 - dimethylamino - 1 - methoxynaphthalene HCl (IV)	6	2	2	23	33
7	1-phenylazo-2-naphthol (V)	1	2	4	25	32
8	1-amino-2-naphthol HCl (VI)	0	3	3	21	27
9	„ „ „ + tin	0	0	1	29	30
10	4-aminodiphenyl (VII)	0	0	1	34	35
11	3-hydroxy-4-aminodiphenyl sulphate (VIII)	4	2	2	30	38
12	4-aminodiphenylyl sodium sulphate (IX)	1	0	0	20	21
13	4′ - nitro - 3 - hydroxy - 4 - aminodiphenyl HCl (X)	0	0	0	20	20

It was decided to assess carcinogenic activity on the incidence of carcinomas. Those carcinomas which did not invade muscle were classified as Grade I, those which were invasive as Grade II. In the course of the histological examination of the material it was observed that those mice implanted with pellets containing chemicals with the highest carcinogenic activity yielded the most widespread and best established tumours in both grades of malignancy. In the case of 1-amino-2-naphthol hydrochloride and 1-phenylazo-2-naphthol, but not of 2-amino-1-naphthol hydrochloride or 3-hydroxy-4-amino diphenyl sulphate, the significant yield of carcinomas was accompanied by a significant yield of benign tumours (Tables II–V). In no case was a significant yield of benign tumours found with an inactive compound. The incidence of benign tumours, and to a lesser extent

TABLE II.—*Incidence of Bladder Changes in Mice Implanted with Paraffin Wax Alone*

Experiment.	No. of mice.	Concretions.	Squamous metaplasia.	Benign tumours.	Carcinomas. I.	Carcinomas. II.	Carcinomas. Total.
1*a*	10	1	0	0	0	0	0
b	27	3	4	2	1	0	1
c	19	2	5	1	0	1	1
Total	56	6	9	3	1	1	2

TABLE III.—*Incidence of Bladder Changes in Mice Implanted with Paraffin Wax and Derivatives of Naphthalene*

Experiment.	Compound.	No. of mice.	Concretions.	Squamous metaplasia.	Benign tumours. Total.	Benign tumours. *P.*	Carcinomas. I.	Carcinomas. II.	Carcinomas. Total.	Carcinomas. P.
2	2-naphthylamine	89	10	16	8	0·32	4	4	8	0·18
3	2-amino-1-naphthol HCl	118	10	30	8	0·50	9	11	20	0·00
4	2-amino-1-naphthol HCl (+tin)	68	2	4	5	0·46	2	1	3	0·57
5	2-amino-1-naphthyl hydrogen sulphate	38	0	5	3	0·47	1	1	2	0·53
6	2-dimethylamino-1-methoxy-naphthalene HCl	33	1	9	6	0·060	1	1	2	0·48
7	1-phenylazo-2-naphthol	32	3	5	6	0·055	8	0	8	0·00
8	1-amino-2-naphthol HCl	36	5	18	8	0·019	7	3	10	0·00
9	1-amino-2-naphthol HCl (+tin)	30	2	20	6	0·043	2	5	7	0·00

P = Probability evaluated by the exact method for 2 × 2 tables (Fisher, 1950).

TABLE IV.—*Distribution of Bladder Changes in Mice Implanted with Paraffin Wax and 2-Naphthylamine or 2-Amino-1-Naphthol Hydrochloride According to the Method of Preparation*

Experiment.	Compound.	Preparation.	No. of mice.	Concretions.	Squamous metaplasia.	Benign tumours.	Carcinomas. I.	Carcinomas. II.	Carcinomas. Total.	Carcinomas. P.
2*a*	2-naphthylamine	B.D.H.	41	8	7	6	2	2	4	0·20
b	,,	R.C.H.	48	2	9	2	2	2	4	0·27
3*a*	2-amino-1-naphthol HCl	A	40	2	11	3	5	4	9	0·00
b	,,	B	43	5	13	4	3	4	7	0·03
c	,,	C	35	3	6	1	1	3	4	0·15
4*a*	2-amino-1-naphthol HCl (with tin)	A	17	1	0	0	1	1	2	0·23
b	Ditto	B	20	0	3	3	1	0	1	0·61
c	,,	C	31	1	1	2	0	0	0	—

P = Probability evaluated by the exact method for 2 × 2 tables (Fisher, 1950).

TABLE V.—*Incidence of Bladder Changes in Mice Implanted with Paraffin Wax and Derivatives of Diphenyl*

Experiment.	Compound.	No. of mice.	Concretions.	Squamous metaplasia.	Benign tumours. Total.	Benign tumours. *P.*	Carcinomas. I.	Carcinomas. II.	Carcinomas. Total.	*P.*
10	4-aminodiphenyl	35	4	5	0	—	0	3	3	0·29
11	3-hydroxy-4-aminodiphenyl sulphate	38	0	9	1	—	7	2	9	0·00
12	4-amino-3-diphenylyl sodium sulphate	21	1	3	1	—	0	1	1	0·62
13	4′-nitro-4-amino-3-hydroxy diphenyl HCl	20	1	8	0	—	0	3	3	0·11

P = Probability evaluated by the exact method for 2 × 2 tables (Fisher, 1950).

of squamous metaplasia, was regarded as valuable supporting evidence for the carcinogenic activity of these compounds.

The results obtained by implanting wax pellets without any added chemical are given in Table II (Experiment 1*a* has been previously reported; Bonser *et al.*, 1952). The results obtained by implanting pellets containing derivatives of naphthalene are given in Table III (7 mice in Experiment 2, and 9 in Experiment 3 have been previously reported). The yield of carcinomas obtained with 2-naphthylamine was not affected by the method of purification of the compound (Table IV). The total yield (8 carcinomas in 92 mice—8·7 per cent) was not significantly greater than that obtained with paraffin wax alone (2/57—3·5 per cent) but histologically the tumours were better established so that 2-naphthylamine must be regarded as possessing a low degree of carcinogenic activity.

2-Amino-1-naphthol hydrochloride (20/118—17·0 per cent) (Table III) is a potent carcinogen. From Table IV it will be seen that whereas the compound prepared by Method A (9/40—22·5 per cent) and by Method B (7/43—16·3 per cent) induced significantly more tumours than did the controls, the compound prepared by Method C (4/35—11·5 per cent) did not. It is possible that this paradoxical result may be explained by the small numbers involved in these experiments, as the deficiency in the group treated with 2-amino-1-naphthol prepared by Method C is of two tumours only. A further group of mice will be implanted to test the validity of this explanation. Alternatively, application of the χ^2 test to the incidence of tumours with 2-amino-1-naphthol hydrochloride shows that such a distribution between the three groups might well occur by chance in experiments of this nature ($\chi^2 = 1{\cdot}7$, $n = 2$, $P = 0{\cdot}45$). Alteration of the 2-amino-1-naphthol molecule by substitution on the oxygen atom as in 2-amino-1-naphthyl hydrogen sulphate (III) (2/38—5·3 per cent) or on both oxygen and nitrogen atoms as in 2-dimethyl-amino-1-methoxynaphthalene hydrochloride (IV) (2/33—6·1 per cent) suppressed the carcinogenic activity.

1-Amino-2-naphthol hydrochloride (10/36—27·8 per cent) is a potent carcinogen under the conditions of bladder implantation. The activity of this compound was not suppressed by recrystallisation in the presence of stannous chloride (7/30—23·3 per cent). 1-Phenylazo-2-naphthol (8/32—25 per cent) is also carcinogenic to the bladder epithelium when locally applied.

The results obtained with pellets containing 4-aminodiphenyl and its derivatives (Table V) are very similar to those obtained with the analogous naphthalene compounds. Although the tumour yield with 4-aminodiphenyl (3/35—8·6 per cent) was not significantly different from that with paraffin wax alone the tumours were much better established. 3-Hydroxy-4-aminodiphenyl sulphate (9/38—23·7 per cent) is a potent carcinogen whereas when the oxygen atom is substituted in 4-amino-3-diphenylyl sodium sulphate (1/21—4·8 per cent) the carcinogenic activity is suppressed. A preliminary group of twenty mice implanted with pellets containing 4′-nitro-4-amino-3-hydroxydiphenyl hydrochloride (X) yielded three well established and invasive carcinomas (15 per cent). Thus, this compound is apparently carcinogenic.

DISCUSSION.

The present experiments confirm the value of the technique of the implantation of paraffin wax pellets containing suspected substances into the lumen of the bladder of the mouse as a test for carcinogenic activity. The chemicals tested fall into

three groups; (i) the four ortho hydroxy-amines, 2-amino-1-naphthol hydrochloride (II), 1-amino-2-naphthol hydrochloride (VI), 3-hydroxy-4-aminodiphenyl sulphate (VIII) and probably 4′-nitro-3-hydroxy-4-aminodiphenyl hydrochloride (X) and also the food colorant 1-phenylazo-2-naphthol (V) are carcinogenic; (ii) 2-amino-1-naphthyl hydrogen sulphate (III), 2-dimethylamino-1-methoxynaphthalene hydrochloride (IV), and 4-amino-3-diphenylyl sodium sulphate (IX) are inactive, while (iii) 2-naphthylamine (I) and possibly 4-aminodiphenyl (VII) occupy an intermediate position.

The occurrence of tumours with paraffin wax alone may be due to the presence of a foreign body in the mouse bladder or to a low degree of carcinogenic activity in the wax used. The latter seems possible as Pullinger (personal communication) has implanted pellets of another batch of wax with and without added material into the bladders of mice and has only obtained one papilloma in 37 mice surviving 25–106 weeks. As in the present experiments the incidence of tumours with the paraffin wax was 3·5 per cent, and with the ortho hydroxyamines was only 15–30 per cent, groups of not less than thirty mice were necessary to give a significant yield of tumours. Groups of 200–250 mice would be necessary to establish the significance of the results with 2-naphthylamine and 4-aminodiphenyl, with which the tumour incidence was only about 8 per cent.

The tumours obtained with 2-naphthylamine are not likely to be due to an impurity in the chemical as the incidence of tumours on implantation with 2-naphthylamine purified by distillation and recrystallisation (Bonser, 1943) and by gradient sublimation (Henson *et al.*, 1954) are similar. Bonser, Clayson, Jull, and Pyrah (1956) showed that when 2-naphthylamine was allowed to stand in oily solution it slowly developed the ability to induce sarcomas when injected subcutaneously into mice. It is probable that a similar mechanism would account for the 2-naphthylamine tumours in the present experiments.

Demonstration of the carcinogenic activity of the ortho hydroxy-amines 2-amino-1-naphthol, 1-amino-2-naphthol and 3-hydroxy-4-aminodiphenyl under the conditions of bladder implantation strongly supports the hypothesis that aromatic amines induce cancer by virtue of their transformation in the body to ortho hydroxy-amines. This activity may be due to (i) direct reaction of the ortho hydroxy-amines with the tissue constituents, (ii) conversion of the ortho-hydroxy-amines to the " true carcinogens " by the enzymes of the tissues, or (iii) conversion of the ortho hydroxy-amines to the " true carcinogens " chemically in the pellet or urine. Even if the latter mechanism is operative it is likely that the traces of free ortho hydroxy-amines now known to be present in the urine of animals treated with the aromatic amines, will undergo similar conversions. The explanation of the lack of activity of the tin complex of 2-amino-1-naphthol and the full activity of the similar complex of 1-amino-2-naphthol requires explanation.

It has been shown that 1-phenylazo-2-naphthol (V) as well as 1-amino-2-naphthol hydrochloride is carcinogenic to the mouse bladder. It is possible that reduction of 1-phenylazo-2-naphthol takes place in the bladder epithelium. Ross and Warwick (1955) have shown that a series of azo compounds are reduced by the xanthine-oxidase-xanthine system and this and similar enzymes are present in many tissues. The local biological activity of 1-phenylazo-2-naphthol has also been demonstrated by Green (personal communication) who has shown it to be a very active tumour inhibitor in rats, when applied by subcutaneous injection.

It was originally suggested that 2-amino-1-naphthyl hydrogen sulphate might be broken down in the bladder to the ortho hydroxy-amine (Bonser *et al.*, 1952). The lack of carcinogenic activity with the sulphate esters contraindicates this possibility, as does the observation of Boyland, Manson, Sims and Williams (1956) that some ortho aminophenyl hydrogen sulphates are not hydrolysed by known mammalian sulphatases. They suggest that free ortho hydroxy-amines are produced in the urine by the enzymic hydrolysis of the corresponding ortho aminophenyl glucuronides or phosphates.

The results obtained by the bladder implantation method require confirmation by other methods. Conventional techniques such as painting and feeding are not likely to be reliable because of the easy oxidation of these compounds, and because an optimum concentration is required at the site of tumour induction. Subcutaneous injection of 2-amino-1-naphthol hydrochloride (Bonser *et al.*, 1952) produced a small yield of local sarcomas while Hueper (1938) obtained retrothelial sarcomas in mice by intraperitoneal injection of the crude chemical. Recently Miller and Miller (1955) attempted to induce tumours in rats by feeding 4-acetamidodiphenyl, 3-hydroxy-4-acetamidodiphenyl and 3-hydroxy-4-aminodiphenyl to rats. They obtained a number of fibroadenomas of the breast during a period of twelve months, at which time they terminated the experiment. Similar lesions were also present in the controls. It is unfortunate that the experiment was terminated at so early a stage as Walpole *et al.* (1952) did not obtain tumours with 4-aminodiphenyl in rats until 570 days.

The present results of testing ortho hydroxy-amines support the hypothesis that aromatic amines are carcinogenic by virtue of their conversion to ortho hydroxy-amines in the animal body. It follows therefore that compounds which may give rise to ortho hydroxy-amines *in vivo* should be regarded as potentially hazardous and that they should not be allowed to come into contact with human beings either in the course of their work or as additives to foodstuffs.

SUMMARY

1. Pellets of paraffin wax implanted into the lumen of the bladder of fifty-seven mice induced two carcinomas.

2. Pellets of paraffin wax containing 2-amino-1-naphthol hydrochloride, 1-amino-2-naphthol hydrochloride, 3-hydroxy-4-aminodiphenyl sulphate and 1-phenylazo-2-naphthol have been shown to be carcinogenic on implantation into the mouse bladder.

3. 2-Naphthylamine and possibly 4-aminodiphenyl have been shown to possess a slight carcinogenic activity and 2-dimethylamino-1-methoxynaphthalene, 2-amino-1-naphthyl hydrogen sulphate, and 4-amino-3-diphenylyl sodium sulphate to be inactive when tested by the technique of bladder implantation.

4. These results support the concept that aromatic amines are carcinogenic because of their conversion in the body to ortho hydroxy-amines.

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TEST OF SOME STEROID SUBSTANCES FOR CARCINOGENIC ACTION IN MOUSE BLADDERS

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SEVERAL exogenous chemical compounds have been found that will induce tumours in the urinary bladder of man and animals. Bladder tumours in workers in the dyestuff industry were traced to the chemical 2-naphthylamine which was shown to be carcinogenic for the bladders of dogs following either injection or oral administration (Hueper, Wiley and Wolfe, 1938). In mice bladder tumours were induced by Armstrong and Bonser (1944) and Foulds (1947) with 2-acetylaminofluorene by feeding, by Holsti and Ermala (1955) unexpectedly by swabbing lips and oral mucosa with tobacco tar ; in rats by Wilson, De Eds and Cox (1941) with acetylaminofluorene, by Dunning, Curtis and Segaloff (1947) and Dunning and Curtis (1952) with subcutaneous pellets of stilboestrol associated with calculus formation in the bladder, and by Walpole, Williams and Roberts (1955) with dimethyl-4-aminodiphenyl. It has been suggested that in every case of bladder cancer whether of exogenous or endogenous origin the tumour arises as a result of a chemical substance in the urine acting locally and directly on bladder epithelium. The possibility of such direct local action was tested by Bonser, Clayson and Jull (1951) using Jull's technique (1951) of implantation of wax pellets impregnated with the test substance into the bladders of mice. They obtained bladder tumours with methylcholanthrene and with a metabolite of 2-naphthylamine.

An earlier attempt in this laboratory to find an endogenous carcinogen in urine of patients with bladder cancer was without result (Chalmers, 1951). The present experiments were designed to test concentrated and prolonged local action of the same ketosteroid fraction and other steroids in wax pellets in mouse bladders. One tumour only arose after 18 months in the series of implants with crude ketosteroid from a case of bladder cancer. Parallel experiments were carried out with extracts of the neutral steroid fraction of human bladder and prostatic cancer urines (Urines A and B respectively).

During the course of these investigations the rate of elimination of the ketosteroid from the pellets in the bladders was followed and results compared with some known steroids, cholesterol, dehydroisoandrosterone and oestrone (Chalmers and Pullinger, 1955). The long term effects of crude ketosteroid and of oestrone were tested deliberately and of cholesterol incidentally, since it was found in unused ketosteroid pellets from Urine B. In Urine A series values up to 0·8 per cent were found in 10 pellets examined after death of the mice.

EXPERIMENTAL

Neutral steroid extracts.—Urine from 2 hospital cancer patients was collected for several days. The pooled urine was hydrolysed and the neutral steroid fraction separated by methods similar to those described by Dobriner, Lieberman and Rhoads (1948). Urine from a bladder cancer patient collected for 8 days gave 318 mg. crude steroid while that from a prostatic patient collected for 9 days gave 214 mg.

Preparation of pellets.—The crude urinary steroid extract together with an equal weight of paraffin wax (m.p. 53°) was dissolved in a small volume of chloroform and aliquots pipetted into cold water to give pellets weighing approximately 10 mg. Control wax pellets were made in a similar manner. Pellets made in this way were used in the early experiments but subsequently when pure chemicals were being tested it was necessary to alter the method of preparation. Dehydroisoandrosterone and cholesterol pellets were made by pouring a chloroform solution containing wax in addition to the steroid into a stainless steel mould, the pellets being dried overnight in a vacuum desiccator. In the case of oestrone, because of its low solubility in organic solvents, pellets were made in a press, weighed, and then coated with wax.

Analysis of Pellets

In the case of the single tumour found in this investigation the pellet when removed from the mouse bladder post mortem was found to be encrusted with inorganic phosphates. For comparative purposes examinations have been made of incrustations or bladder stones found post-mortem in these experiments. With regard to the incrustations, the pellet after removal from the bladder was weighed and then dissolved in hot ethanol or in the case of cholesterol in chloroform and the insoluble material filtered off, dried and weighed. If sufficient material was present a chemical analysis was made in the usual way (King, 1951*a*) and in such cases calcium, ammonium and magnesium phosphates were found to be present.

17-*Ketosteroid.*—After filtering off any incrustation from the ethanol solution of the pellet, the wax which precipitated on cooling was centrifuged off. The ketosteroid in an aliquot of the supernatant was estimated by the method of Drekter, *et al.* (1952) in which the colour with *m*-dinitrobenzene is measured.

Cholesterol.—The weighed pellet was disolved in chloroform and estimated colorimetrically using acetic anhydride and conc. H_2SO_4, as described by King (1951*b*).

Oestrone.—The pellet was weighed and dissolved in about 70 ml. ethanol by heating and shaking for 15 minutes. After making up the volume to 100 ml. an aliquot of 1 ml. was taken for analysis. Oestrone was estimated by Brown's method (1952) using the quinol and aqueous sulphuric acid reagent as modified by Bauld (1954).

Implantation of Pellets

Male RIIIf mice about 3 to 4 months old were used because available and proven susceptible to bladder cancer (Armstrong and Bonser, 1944). Jull's (1951) technique for implantation was slightly modified to enable it to be carried

out with the least assistance. The head of the mouse was placed nearest the operator. A small piece of sterile linen with a transverse slit in it was laid over the exposed bladder and the bladder then pulled gently through the slit. The linen was then clipped to the cloth on the operating table so as to stretch the slit slightly and thus hold the bladder in position. The linen served as well to sop up urine when the bladder was incised. Before cutting the bladder a holding stitch was put in near the site of the incision about to be made. It was held during the operation by Spencer Wells forceps laid on the table. This became the first stitch for sewing up after insertion of the pellet. A small cut was then made in the bladder. The whole of this operation can be done by one operator. The mice were anaesthetised subcutaneously with a suspension of bromethol in saline.

Results of long term implants

1. *Ketosteroid Urine A.*—Implants were made into 20 mouse bladders. 16 mice survived for 25 weeks or longer and 13 of these from 12 to 25 months. Only one bladder tumour was found 18 months after operation. It was a multilobular, sessile, transitional-celled papilloma. Urethral concretions were present but there was no obstruction. The pellet was pigmented, slightly coated and impregnated with salts and its cholesterol content was 0·8 per cent.

2. *Ketosteroid Urine B.*—Implants were made into 17 mouse bladders. 6 mice survived for 20 to 23 weeks. All the remainder died after 25 to 26 weeks from an epidemic of Tyzzer's disease. No bladder tumours were seen.

3. *Oestrone pellets.*—Implants were made into 26 mouse bladders. 16 mice survived for 25 weeks or longer but none for more than 10 months. No bladder tumours were found. All deaths at and after 25 weeks were associated with over-distension of bladders, incontinence and hydronephrosis. The pellets were not moulded into urethral orifices. The bladder distension and hydronephrosis were attributable to atony of bladder musculature induced by oestrogen. Further evidence of absorption of oestrone through the bladder epithelium was obtained by examining the mammae. An average of 5 to 6 rudiments had developed in all these male mice. They were hyperplastic and ducts and alveoli contained a milk-like secretion as is characteristic for this strain in response to natural oestrogens. A few pituitaries were enlarged.

4. *Control wax pellets.*—Implants of wax pellets alone were made into 38 mouse bladders. Thirty-four mice survived to 25 weeks or more and 25 of these from 12 to 25 months. Twelve are still alive after 16 months.

All pellets became coated and impregnated with inorganic salts (calcium, magnesium, ammonium phosphates) but only 3 were appreciably enlarged to form calculi. These were all control wax pellets which had been in the bladders from 1 to 9 months ; 2 were moulded with projections into urethral orifices and had caused obstruction and hydronephrosis. Many pellets became pigmented besides those containing the coloured crude ketosteroid. Some were folded on themselves. No calculi were found in any steroid containing pellets. In a few bladders multiple small calculi had formed. The shape and size of the oestrone pellets were not altered but these were coated as usual with salts. The bladder distension and overflow incontinence associated with them was not accompanied by mechanical obstruction.

Urethral concretions were found in mice with control wax and with ketosteroid pellets. They did not give rise to urinary obstruction. One example of pyonephrosis was seen.

RESULTS

Rate of disappearance of steroids from Pellets

1. *Ketosteroid*

Urine of bladder cancer patient (*A*).—20 pellets weighing about 10 mg. each were inserted. Initially the ketosteroid content of the pellet was 5 per cent, each pellet containing about 0·5 mg. Four of the mice died within 5 days; others lived for 5½ months or more and several for about 2 years. Ketosteroid estimations were carried out on pellets recovered from the bladders of mice post mortem. It was found that the ketosteroid was eliminated completely after 6 to 7 months and in 2 cases negative values were found after 2 and 5 days respectively.

Urine of prostatic cancer patient (*B*).—Seventeen pellets were inserted, each weighing approximately 10 mg. and containing 0·4 mg. ketosteroid, which was equivalent to 4 per cent. Sixteen lived more than 5 months but owing to an intercurrent infection all were dead after 6 months. As in the previous case the elimination of the ketosteroid from the mouse bladder was followed. In 7 cases the steroid was completely eliminated after 5½ to 6 months, but in 3 cases after 6 months values of 1 to 1·7 per cent were found.

Dehydroisoandrosterone.—In view of the relatively rapid disappearance of the ketosteroid from the wax pellets made from urine extracts when implanted in the mouse bladder, an examination was made of the elimination of dehydroisoandrosterone. This compound, which had been used in standardising the ketosteroid estimation, was made into pellets with wax and tested in the same way as the extracts of urine. The pellets weighed 5 to 10 mg. and contained 20 per cent ketosteroid, i.e. about 1 to 2 mg. The results are recorded in Table I.

2. *Cholesterol*

Initially the cholesterol content of pellets made from steroid extract of Urine B (prostate patient) was 1·9 per cent, i.e. 0·19 mg. per pellet. After 24 months in the mouse bladder the cholesterol remaining in the pellet was 0·4 per cent. In the case of urine from Patient A (bladder) the cholesterol content of the pellet after 14 and 18 months was 0·5 and 0·8 per cent respectively.

Eik-nes, Schellman, Lumry and Samuels (1954) showed that cholesterol was less soluble in buffer solution than other steroids including dehydroisoandrosterone and it was anticipated that its relative insolubility would result in a slower elimination. To confirm this view tests were made with cholesterol pellets. The pellets weighed approximately 10 mg. and each contained about 2 mg. of cholesterol. The findings are shown in Table I.

3. *Oestrone*

The rate of disappearance of oestrone from pellets inserted into mouse bladders has also been examined. As already mentioned, owing to its insolubility this compound could not be made into pellets in the usual manner. Weighed pellets

of oestrone however were coated with wax and used in this way. The pellets weighed 5 to 17 mg. and contained 4 to 13 mg. oestrone. As might be expected oestrone was removed relatively slowly from the mouse bladder (Table I).

TABLE I.—*Rate of Disappearance of Steroids from Pellets*

Duration of experiment in days.	Percentage residual.		
	Dehydroiso-androsterone.	Cholesterol.	Oestrone.
2	78	—	91
3	75	100	—
5	72	—	—
6	—	97	98
32	—	—	97
33	—	97	—
35	20	—	—
62	—	96	—
63	11	—	82
89	3	78	—
90	—	—	85
116	—	—	77

COMMENT

As a test of ketosteroid action in the bladder the experiment cannot be considered satisfactory on account of the rapid elimination from the pellet. Mice containing oestrone pellets survived too short a time. Evidence of absorption of oestrone from the pellet through the bladder epithelium was unexpected. The possibility that cholesterol might be carcinogenic to bladder epithelium was considered in the light of Hieger's evidence (Hieger, 1949 ; Hieger and Orr, 1954), that it can induce sarcoma in mouse connective tissue. The single papilloma occurred in 1 of 10 mice with crude ketosteroid pellets containing cholesterol which survived for 16 to 25 months, compared with none in 17 with wax alone. An adequate test could only be done with pure cholesterol and larger numbers of mice.

SUMMARY

Crude ketosteroid fractions from urine of human cancer patients were tested for carcinogenic action in pellets in mouse bladders. The ketosteroids were rapidly eliminated. One papilloma only was found.

Mice implanted with oestrone pellets did not survive longer than 10 months. Oestrone was absorbed from the bladder pellets. No tumours were obtained.

Paraffin pellets alone did not cause tumours.

We are indebted to Mr. Wm. S. Mack and Mr. J. H. McBeath for arranging for the collection of urine from their patients, and to Mrs. R. H. Jack for help with the chemical analyses.

ADDENDUM.

The 12 remaining control mice died or were killed, between 17 and 20 months. None had bladder tumours.

In a paper published as this article was finished, Boyland and Watson (1956) have recorded the production of bladder papilloma and carcinoma with an endogenous metabolite of tryptophan, 3-hydroxy-anthranilic acid, in bladder pellets. Cholesterol tested in the same way gave negative results.

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ANTIGENIC DIFFERENCES BETWEEN NORMAL HAMSTER KIDNEY AND STILBOESTROL INDUCED KIDNEY CARCINOMA: COMPLEMENT FIXATION REACTIONS WITH CYTOPLASMIC PARTICLES

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It has been shown previously (Weiler, 1952*a*, 1952*b*) that the organ specific antigen of rat liver cytoplasmic particles could not be found in primary hepatoma induced by 4-dimethylaminoazobenzene. Hepatome particles, on the other hand, did contain antigenic properties which were not present in normal liver, which could, however be found in other organs of the rat.

It seemed important to know whether these qualitative antigenic changes during carcinogenesis are a peculiar characteristic of the liver tumour, or whether they are of more general significance in tumour formation.

The stilbestrol induced kidney carcinoma of the golden hamster seemed to provide suitable material for the comparison of malignant tissue with its homologous normal tissue as to their antigenic properties. The tumour is known to arise from the tubular epithelium of the kidney cortex (Horning and Whittick, 1954). On the other hand, organ specific antigenic properties have been shown to be present in the kidney cytoplasmic particles (Henle, Chambers and Groupé, 1941; Furth and Kabat, 1941). It had to be found, therefore, whether the kidney tumour cytoplasmic particles contain kidney specific antigen, and whether in the tumour tissue a new antigen might be present which is not contained in normal kidney.

MATERIALS AND METHODS

Tumours

The hamster strain of the Chester Beatty Institute was used throughout. Tumours arose in the kidneys 8–12 months after the implantation of stilboestrol pellets, as studied in detail by Horning (1954) and Horning and Whittick (1954). The size of the tumours which were used for the preparation of cytoplasmic particles varied from 5 to 15 mm. in diameter. Only tumour tissue which appeared to be non-necrotic was used.

Preparation of cell fractions.—Tumour or whole normal kidneys, freed from their capsule, were used either fresh, directly after dissection from the animal, or after up to several weeks storage at − 35° C; in the latter case, tissues had been quickly frozen in test tubes immersed in dry ice-alcohol before being transferred to the deep-freeze cabinet.

The tissues were homogenized in 9 volumes buffered sucrose solution (0·25 M sucrose, M/100 phosphate buffer pH 7·3) by means of a Potter-Elvehjem glass

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homogenizer. The homogenates were layered in centrifuge tubes over a sucrose solution of somewhat higher molarity, and spun at 2000 *g.* for 8 min. The sediment (" nuclear fraction ") was usually resuspended and centrifuged a second time, the second supernatant then being combined with the first. The combined supernatants were centrifuged for 10 min. at 10,000 *g.* in an MSE refrigerated centrifuge, yielding the mitochondrial fraction as sediment. The remaining supernatant was spun either for 30 min. at 60,000 *g.* in a Spinco L centrifuge, or for 90 min. at 20,000 *g.* in the high speed head of the International refrigerated centrifuge. The sediment so obtained was the microsome fraction. Both microsomes and mitochondria were washed once by resuspending and centrifuging again at the same speed. The fractions were suspended in buffered sucrose solution, and either used fresh for serological tests, or stored at — 35° C. after quick-freezing in solid CO_2-alcohol mixture. All operations were carried out in the cold.

Antisera

Rabbits were injected three times weekly, with increasing amounts of antigen, by the intravenous route. The final two injections were given intravenously and intraperitoneally as well. The injection procedure lasted 3 to 4 weeks, and a total of 3 to 6 mg. antigen nitrogen were given to each rabbit.

Absorption experiments were carried out using cytoplasmic particles from kidney, liver, lung, and sheep red cells as absorbing antigens. Except for kidney, where combined microsome and mitochondrial fractions were used, the particles for absorption experiments were prepared by centrifugation for 80 min. at 20,000 *g.*, after previously removing the nuclear fraction at low speed. Thus, the particle preparations represented mixtures of microsomes and mitochondria. In the case of lung, the nuclear fraction was sometimes also used, in addition to cytoplasmic particles, as absorbing antigen.

For the actual absorptions, antiserum and particles were incubated at 37° C. for 30 minutes, and then kept in the refrigerator for 1 or 2 days. Precipitate and particles were removed by centrifugation, and the procedure was repeated, until at least 0·05 ml. gave no complement fixation reaction with the antigen used for absorption ; 4 to 6 absorptions were usually necessary to achieve this, with total amounts of antigen up to 2 mg. antigen N for each ml. of antiserum.

In absorption experiments with sheep cells, about 0·2 ml. of packed cells were added to each ml. of antiserum, kept for 30 min. at 37° C. and then for about 2 hours in the refrigerator. This was repeated once. Thereafter, no Forssman antibody could be detected in the antisera.

Serological tests.—The serological procedure has been described elsewhere (Weiler, 1956). In brief, serial twofold dilutions of either antiserum or antigen were carried out. The activity of antisera was estimated by setting up an antiserum dilution series against a constant amount of 8 μg. N antigen. The activity of a given particle preparation was determined in dilution series of antigen reacting with constant antiserum excess ; the amount of antiserum in these titrations was always five times the minimum amount of serum, which gave at least 3 + reaction in the antiserum dilutions carried out previously. Several checkboard experiments (varying both antiserum and antigen at the same time) have been carried out, using kidney antisera and kidney cytoplasmic particles ; by this it could be secured that the fivefold minimum amount of antiserum, as used in the antigen titrations, really represented antiserum excess.

RESULTS

Antigenic spectrum of cytoplasmic particles from normal kidney

Unabsorbed antisera against kidney microsomes and kidney mitochondria show complement fixation reactions with cytoplasmic particles prepared from several hamster organs ; moreover, they contain Forssman antibodies, as indicated by sheep red cell haemolysis. Exhaustive absorption with liver particles does not eliminate Forssman antibodies nor antibodies reacting with lung particles. After elimination of Forssman antibodies by a subsequent absorption with red cells, there still remain antibodies reacting with lung particles. If these absorptions are followed by a third absorption with lung particles, the antisera react neither with particles from liver or lung nor with those prepared from spleen, heart, or testes. As these antisera still react with kidney particles, they are regarded as kidney specific.

The relative amounts of antibodies in antisera against kidney cytoplasmic particles can be seen from Table I. Judged by the minimal amounts reacting with

TABLE I.—*Minimum Amounts of Antiserum (ml.) Reacting with 8 μg. N of Homologous Antigen. 2 Units of Complement*

	Absorbed with				
	Successively.				Separately.
Antiserum against—	—	Liver.	Sheep cells.	Lung.	Kidney.
KiC	0,00[illegible]125	0,004	0,008	0,01	0,1
	4 +	3 +	3 +	3 +	0
KiT	0,0005	0,002	0,004	0,0025	0,1
	4 +	4 +	4 +	2 +	0
TuC	0,001	0,001	0,025	0,07	0,01
	4 +	3 +	3 +	0	3 +
TuT	0,0005	0,001	0,025	0,05	0,02
	2 +	4 +	3 +	0	3 +

Degree of reaction with endpoint antiserum volume : 4 + = no haemolysis ; 0 = complete haemolysis, 3 + to 1 + = intermediate values.

Abbreviations : KiC = kidney microsomes. KiT = kidney mitochondria
TuC = Tumour microsomes. TuT = Tumour mitochondria.

8 μg. N of homologous kidney, antigen absorption with liver particles removes about 60 per cent of the antibodies, subsequent absorption with sheep cells another 20 per cent, and finally absorption with lung particles another 10 per cent, thus leaving about 10 per cent kidney specific antibodies These figures concern anti-microsome sera ; anti-mitochondrial sera have about 20 per cent of their activity left as kidney specific antibody after the absorption procedure.

The content in particle fractions of antigens with different specificity can be tested by setting up serial dilutions of antigens against antiserum excess. As can be seen from Table II, kidney microsomes show about half the antigenic activity when reacting with kidney specific anti-microsome serum, as when reacting with unabsorbed serum. Mitochondria retain even more of their activity in reactions with absorbed antisera. These experiments indicate that in microsomes

about half of all antigenic groups and in mitochondria even more than half are kidney specific.

There is a pronounced similarity between microsomes and mitochondria as to their reactions with homologous absorbed or unabsorbed antisera. It should be possible to detect differences between both types of particles by cross reactions with the respective heterologous antisera. As can be seen from Table II the reactions of microsomes with anti-mitochondrial sera, or of mitochondria with anti-microsomal sera, are very slightly weaker than the reactions of both types of particles with their homologous antisera. This difference, however slight, was reproducible and somewhat more pronounced in checkboard tests, varying the amounts of both antiserum and antigen in one experiment. From the slight quantitative difference between microsomes and mitochondria, as indicated in these experiments, it can be concluded (as far as the reactions with kidney specific antisera are concerned) that the kidney specific antigen is not quite uniform.

TABLE II.—*Minimum amounts of antigens (μg. N) reacting with various antisera. Antiserum excess*

Antiserum against.	Absorbed with—	Antigen. KiC.	KiT.	TuC.	TuT.
KiC	—	1 (4 +)	1 (3 +)	1 (3 +)	1 (2 +)
	Liver	2 (4 +)	2 (3 +)	2 (4 +)	2 (3 +)
	Sheep cells	2 (3 +)	1 (3 +)	8 (3 +)	8 (4 +)
	Lung	2 (4 +)	1 (2 +)	—	—
KiT	—	1 (3 +)	1 (4 +)	1 (3 +)	1 (3 +)
	Liver	2 (4 +)	1 (3 +)	2 (3 +)	2 (3 +)
	Sheep cells	2 (3 +)	1 (3 +)	4 (2 +)	8 (4 +)
	Lung	2 (3 +)	1 (3 +)	—	—
TuC	—	2 (4 +)	2 (3 +)	0,5 (3 +)	1 (3 +)
	Liver	2 (2 +)	4 (3 +)	2 (4 +)	2 (3 +)
	Sheep cells	2 (2 +)	4 (3 +)	4 (4 +)	4 (2 +)
	Lung	—	—	—	—
	Kidney	—	—	4 (3 +)	4 (3 +)
TuT	—	4 (4 +)	4 (3 +)	0,5 (2 +)	1 (2 +)
	Liver	8 (4 +)	8 (4 +)	2 (4 +)	2 (3 +)
	Sheep cells	8 (4 +)	8 (4 +)	4 (4 +)	8 (4 +)
	Lung	—	—	—	—
	Kidney	—	—	4 (3 +)	8 (4 +)

Sera were absorbed with liver, sheep cells and lung successively, with kidney separately. Degree of reaction with endpoint antigen amounts (in brackets). Compare legend of Table I.—=no detectable reaction.

Antigenic spectrum of cytoplasmic particles from kidney carcinoma

Antisera against tumour particles react with particles from other organs as well. A small part of tumour antibodies can be absorbed with liver particles, leaving a strong Forssman antibody activity in the sera. By subsequent absorption with sheep red cells, a great deal of the over-all activity is removed; only 2 or 4 per cent respectively of the antibodies reacting with homologous antigen remain in the tumour antisera after successive absorptions with liver particles and sheep red cells (Table I). If the antisera—in a third absorption step—are absorbed with lung particles, no significant reaction with homologous antigen remains detectable. This means that the tumour antisera do not contain an antibody corresponding to

the kidney specific antibody in kidney antisera ; for in the latter case the kidney specific antibodies could not be absorbed with particles from liver, lung, and sheep red cells.

In tests with constant a mount of antiserum, serial dilutions of both tumours microsomes and mitochondria give strong reactions with unabsorbed homologous antisera. The reactions with tumour antisera absorbed with liver particles alone or with liver particles and sheep red cells are not uniform. when different tumour preparations are employed. Whereas with unabsorbed tumour antisera microsomes and mitoehondria give strong reactions in 0·5 μg. or 1 μg. quantities, the minimum antigenic dose for 3 + reaction varies between 2 μg. N and 8 μg. N, when the particles are tested against tumour antisera absorbed with liver or liver and sheep cells (Table II).

Cross reactions between kidney and tumour particles

(a) *Kidney antisera.*—With unabsorbed kidney antisera, tumour particles give a somewhat weaker reaction than with their homologous antisera. The reaction grows progressively weaker, when antisera successively absorbed with liver particles and with sheep red cells are employed in the test. With kidney specific antisera, i.e. sera absorbed successively with liver, red cells, and lung particles, neither tumour microsomes nor tumour mitochondria give any reaction, employing antigen doses up to 32 μg. N. That is, the kidney specific antigen could not be found in tumour particles. The tumour nuclear fraction or the soluble supernatant remaining after the sedimentation of the microsomes also gave no reaction with kidney specific antisera.

This result was obtained with all tumour preparations tested, the major part of which consisted of pooled tumours. Freezing and storing of tumour tissue before fractionation, or freezing and storing of cytoplasmic fractions, had no effect on the result of this experiment, as could be demonstrated by parallel tests employing fresh material and material stored in the frozen state.

(*b*) *Tumour antisera.*—Kidney particles give a considerably weaker reaction with unabsorbed tumour antisera than with their homologous antisera. The reaction grows progressively weaker after absorption of tumour antisera with liver particles or with liver particles and sheep red cells (Table II).

To test the possibility that tumour antisera contain " tumour specific " antibodies, absorption experiments were carried out employing kidney particles as absorbing antigen. After exhaustive absorption of antitumour sera with kidney particles, a small fraction of antibodies reacting with tumour particles remained in the sera. In antimicrosome serum, about 10 per cent of the activity remains after absorption with kidney particles. in antimitochondrial serum only about 2·5 per cent (Table I). The experiment was repeated three times, employing different tumour preparations as test antigens, with consistent results.

Tumour antigen titrations, employing constant amounts of kidney absorbed antitumour sera, reveal that the minimum amount of antigen necessary for 3 + reaction was 4 or 8 μg. N. Compared with reactions employing unabsorbed antisera, the " tumour specific " antigenic activity of tumour particles is very weak (Table II).

From these experiments it can be concluded that in tumour cytoplasmic particles there is an antigenic component which is not present in kidney cells. This is, however, not " tumour specific " as all antibodies from tumour antisera can be

eliminated by successive absorptions with heterologous antigens : liver, lung, and sheep red cells.

In all experiments with tumour cytoplasmic particles, microsomes, and mitochondria resembled each other qualitatively, and showed very similar quantitative reactions (Table II).

DISCUSSION

Cytoplasmic particles from kidney carcinoma appear not to contain the organ specific antigen which is present in the microsomes and mitochondria from normal kidney. This result was obtained by complement fixation tests in which tumour particles gave no reaction with organ specific antisera against particles from normal kidney.

Quantitatively, one μg. N of normal particles gave a 4 + reaction, whereas no significant reaction could be obtained with tumour particles, using up to 32 μg. N of antigen. Assumming a quantitative difference rather than a qualitative one, the kidney specific antigen content of tumour cytoplasmic fractions would be at least 64 times lower than that of normal particles. In the latter the specific antigen constitutes about half of the total serological activity.

The most striking result is the complete resemblance in the serological characters between the kidney carcinoma and the butter-yellow induced rat hepatoma (Weiler, 1952*a*, 1952*b*), each compared with their respective homologous organ. In both cases the organ specific antigen of the cytoplasmic particles is lost.

It seems reasonable to postulate, therefore, that in normal cells organ specific antigens are a chemical expression of the morphological and functional differentiation, and part of the organ specific growth regulating system. Their loss during carcinogenesis leads to dedifferentiation and unlimited growth. The " deletion hypothesis " has been discussed in more detail by Miller and Miller (1953), Druckrey (1954), Rusch (1954), and more recently, by Haddow (1955). In future work the chemical nature and physiological significance of organ specific antigens will have to be investigated.

In yet another respect there is a resemblance between hepatoma and kidney carcinoma : both tumours contain in their cytoplasmic particles an antigenic component not present in their homologous normal organs. This could be demonstrated by the presence of antibodies in antitumour sera after complete absorption with particles from the normal organ. The antibodies demonstrated in this way are, however, not "tumour specific ", as they could be absorbed by antigens from heterologous organs ; in the case of kidney tumour antisera, the whole serological activity could be eliminated by successive absorptions with liver particles, sheep cells (Forssman antibody), and lung particles. Compared with the liver-hepatoma system, however, the serologic activity of kidney absorbed antisera as well as the activity of tumour particles reacting with excess of this antiserum is very low. This indicates, that the tumour antigens, which are not contained in kidney, but in heterologous organs, play only a minor role in the antigenic spectrum of the kidney carcinoma cell.

SUMMARY

The antigenic relationships between microsomes and mitochondria from normal hamster kidney and from stilboesterol induced kidney carcinoma have been investigated. Rabbit antisera against cytoplasmic particles from normal and

malignant tissue were used. The antigenic activity of cytoplasmic particles was determined in complement fixation tests.

Antisera against kidney cytoplasmic particles were rendered kidney specific by successive absorptions with liver particles, sheep red cells, and lung particles. Tumour microsomes and mitochondria did not react with kidney specific antisera. This indicates that tumour cells do not contain the organ specific antigen of normal kidney cytoplasmic particles.

The whole activity of tumour antisera could be abolished by successive absorptions with liver particles, sheep red cells. and lung particles. That means that tumour antisera do not contain antibodies corresponding to the kidney specific antibodies.

When tumour antisera were exhaustively absorbed with kidney particles, there remained a weak activity towards the homologous antigen ; tumour particles, therefore, seem to contain a small antigenic component which is not present in normal kidney, which can, however, be found in other organs of the hamster.

With unabsorbed antisera, quantitative differences were detectable between cytoplasmic particles from normal and malignant tissue, when cross reactions with the respective heterologous antisera were carried out.

The antigenic differences between normal and malignant tissue, as reported in the present paper, were very similar to those found previously in liver carcinogenesis.

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ANTIGENIC DIFFERENCES BETWEEN NORMAL HAMSTER KIDNEY AND STILBOESTROL INDUCED KIDNEY CARCINOMA: HISTOLOGICAL DEMONSTRATION BY MEANS OF FLUORESCING ANTIBODIES

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As has been shown in the preceding paper, qualitative differences exist between cytoplasmic particles from normal hamster kidney and from kidney carcinoma. Most pronounced is the lack in tumour particles of the kidney specific antigen. It was attempted to demonstrate this antigenic difference between normal and malignant cells histologically, using fluorescein labelled kidney specific antibody, which was expected to yield—as a specific serological reaction—a fluorescent staining with normal kidney cells, but not with malignant cells. Moreover, it was hoped to detect, by this method, cells in early stages of tumour formation by their different degree of staining as compared with normal cells. This has been possible in rat liver carcinogenesis (Weiler, 1956).

MATERIAL AND METHODS

The tumours used were the same as in the preceding paper. Slices 2—3 mm. in thickness, containing both tumour and normal kidney tissue, were prepared as soon as possible after sacrificing the animals. They were immediately placed in specimen tubes which were then immersed in solid CO_2-alcohol-mixture. The tubes containing the tissue slices were then stored at — 35° C. until use.

Sections 5 μ thick were prepared in a modified Linderstrom-Lang cryostat, which was cooled by dry ice, as has been described previously (Weiler, 1956).

EXPLANATION OF PLATES

FIG. 1, 3, 4, 6, 8, and 10 are fluorescence micrographs of fresh frozen sections treated with fluorescein coupled antiserum globulin.

FIG. 1.—Normal kidney stained with kidney specific antibody. 2 glomeruli in the upper part. × 58.

FIG. 2.—Same field as Fig. 1, haematoxylin-eosin.

FIG. 3.—Normal kidney stained with kidney specific antibody. Proximal convoluted tubules. Glomerulus in the upper right. × 230.

FIG. 4. —Edge of a carcinoma stained with kidney specific antibody. × 56.

FIG. 5.—Same field as Fig. 4, haematoxylin-eosin.

FIG. 6.—Small carcinoma nodule stained with kidney specific antibody. × 56.

FIG. 7.—Same field as Fig. 6, haematoxylin-eosin.

FIG. 8.—Edge of a carcinoma stained with carcinoma antibody. 4 glomeruli. × 56.

FIG. 9.—Same field as Fig. 8, haematoxylin-eosin.

FIG. 10.—Early neoplastic foci (arrow), stained with kidney specific antibody. 2 glomeruli. × 56.

FIG. 11.—Part of the field of Fig. 10 at higher magnification, haematoxylin-eosin. × 112.

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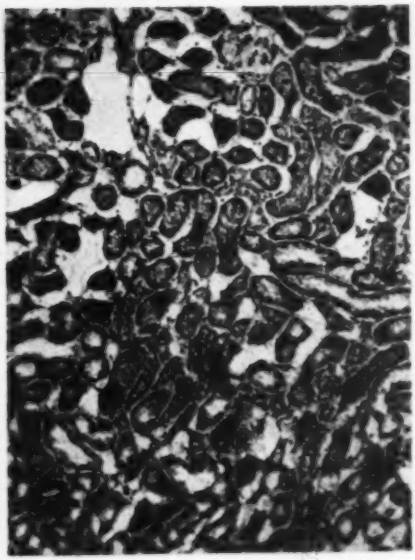

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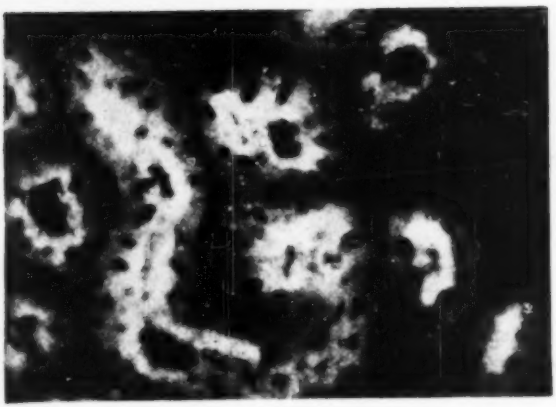

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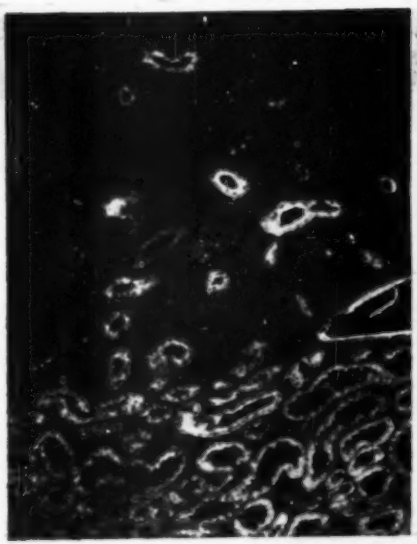

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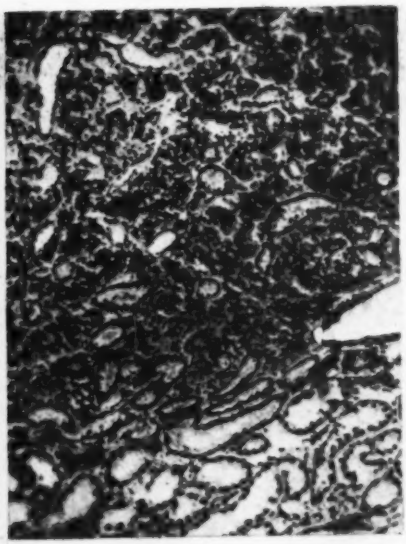

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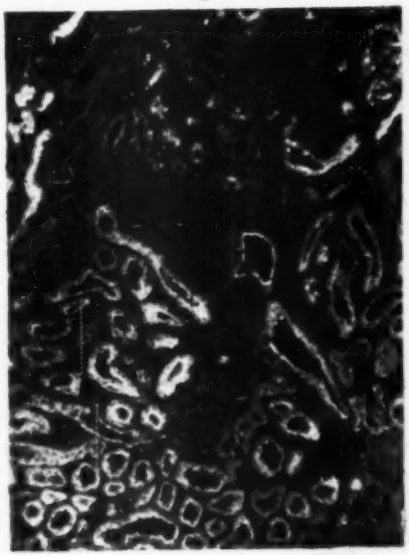

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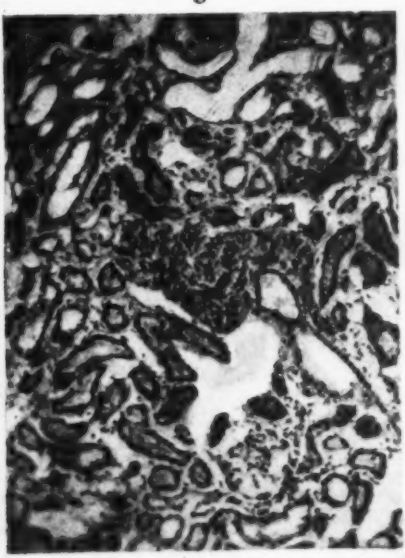

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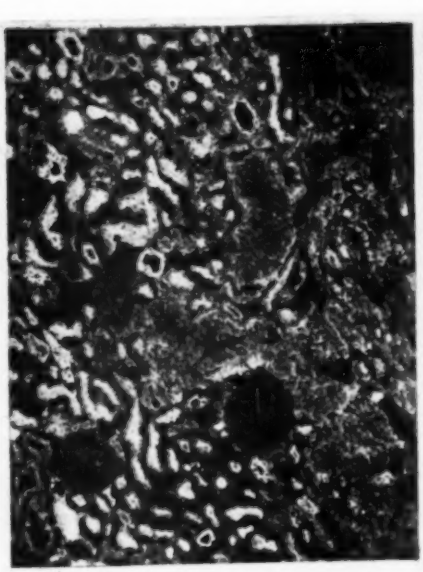

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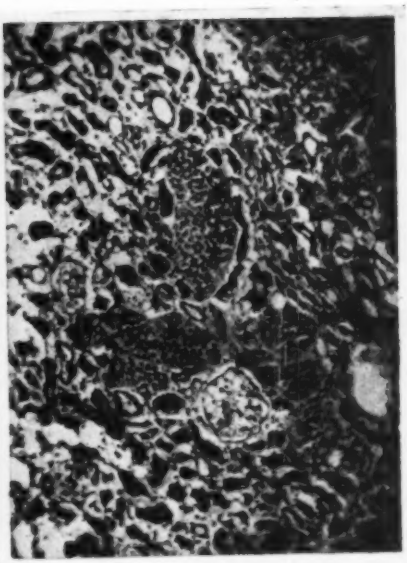

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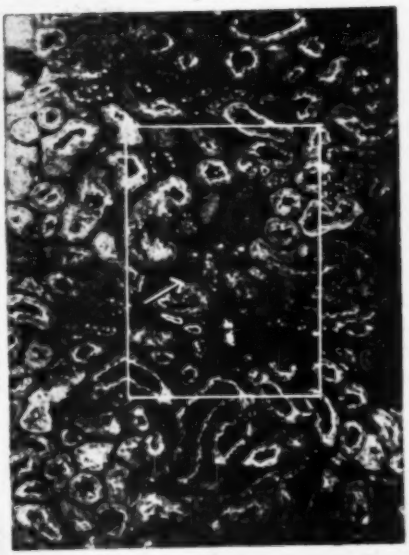

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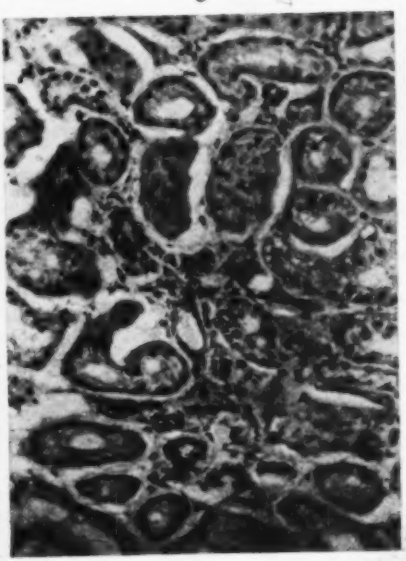

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The following modification was introduced in the technique : after the sections had thawed outside the cryostat, they were immediately—while still wet—placed in absolute alcohol, which had been chilled to — 70° C. by immersing the staining jar in dry ice alcohol mixture. The sections remained in chilled alcohol for about half an hour (thus undergoing a process of " freeze-substitution "), and were then dried under a fan in a cold room at + 3° C. This technique proved to be advantageous for preserving the histological structure of the tissue. Subsequent sections after thawing were routinely fixed in Bouin and stained with haematoxylin-eosin.

For coupling with fluorescein isocyanate combined microsomal and mitochondrial antisera were used. The sera were mixed in equal parts. The preparation of fluorescein-isocyanate and the coupling of the antisera was carried out exactly as described by Coons. Nonspecific staining material was eliminated from the coupled antisera by absorption with acetone dried rat liver powder. In the case of kidney specific antisera, this absorption with acetone dried material proved to be unnecessary, when the sera had been absorbed with liver particles, sheep cells, and lung particles, after the coupling with fluorescein isocyanate. Staining and mounting was carried out exactly as described by Coons.

In fluorescence microscopy, a Mazda 250 watts high-pressure mercury arc was used as light source. The following filters were used : Corning 8440 (2 mm.), and 10 per cent $CuSO_4$ (10 mm.) between light source and microscope ; Leitz Euphos (2 mm.), and a light green filter (Kodak) as eye-piece filters. For low-power photographs the quartz condenser was fitted with a central field stop to obtain dark field effect, and for high-power photographs a Cooke dark field condenser was used.

The following controls were carried out : (*a*) reactions with fluorescein coupled normal rabbit serum globulin ; (*b*) pretreatment of the sections with uncoupled antiserum, thereafter staining with fluorescein antiserum ; (*c*) treatment of heterologous sections (liver) with kidney specific antiserum. In controls (*a*) and (*c*) no green fluorescence was visible ; control (*b*) showed a trace of fluorescence. The specificity of the fluorescent staining reactions was thus ensured.

RESULTS

Reactions with normal kidney antiserum

(a) *Normal kidney.*—There was only a slight difference in the fluorescent microscopic appearance, whether the sections were stained with unabsorbed or with kidney specific antiserum. With the specific serum, the staining was entirely confined to the tubular epithelium, whereas unabsorbed antiserum gave a trace staining reaction with glomeruli, too. With both sera, the proximal convoluted tubules showed the highest intensity of fluorescence. The distal convoluted tubules showed nearly as strong a fluorescence, while a weaker reaction was found in the collecting tubules and the Henle loops.

In hamster kidney the normally occurring " pigment granules ", as described in detail by Sjöstrand, occur very infrequently.

The most surprising result was that most of the fluorescent material was concentrated in the brush border part of the proximal convoluted tubules (Fig. 3). In the distal convoluted tubules the fluorescence was more scattered throughout the cytoplasma, though a certain accumulation of fluorescent material towards

the lumen could be observed in these tubules too. In the Henle loops, and the collecting tubules, the fluorescent staining was more uniform.

Cell nuclei did not show a definite staining. A faint fluorescence could be seen in the nuclei, but this seemed to be due to over- or underlying cytoplasm (Fig. 3).

(b) *Tumour tissue.*—In accordance with the complement fixation reactions using isolated particles the tumour cells did not show any reaction with fluorescent kidney specific antibody. (Fig. 4, 5, 6, 7.) This result was verified with 8 different tumours. Due to the infiltrative growth of the tumour, kidney tubules, completely surrounded by tumour tissue could frequently be seen (Fig. 4, 5).

Early stages of tumour formation were often observed in kidneys from stilboesterol treated hamsters. Histologically, they appear as foci having about the diameter of a normal kidney tubule, and consisting of very basophilic small cells. In sections treated with the fluorescent kidney specific antibody. these foci do not show any green fluorescence. They are already devoid of kidney specific antigen. However, very frequently they contain granules having a bright orange-yellow fluorescence (Fig. 10).

With unabsorbed kidney antiserum, there was a staining reaction with tumour cells, although much weaker than with normal kidney cells.

Reactions with tumour antisera

As the complement fixation reaction with kidney absorbed tumour antiserum was only very weak, no attempt was made to use the absorbed serum for fluorescence work.

Unabsorbed tumour antiserum gives a strong fluorescent staining reaction both in tumour tissue and in kidney tubules. Glomeruli show a much weaker but definite reaction (Fig. 8, 9). The tumour nuclei remain unstained, giving a sponge-like appearance of the tumour itssue.

DISCUSSION

The qualitative antigenic difference between cytoplasmic particles from normal kidney and from stilboestrol induced kidney carcinoma, which had been established by complement fixation tests (Weiler, 1956), could also be demonstrated using fluorescein labelled antibodies. Fluorescent kidney specific antiserum globulin reacts in fresh frozen histological sections with the cytoplasm of the kidney tubular cells, but not with tumour cells.

Unabsorbed antiserum against kidney particles gave a relatively weak reaction with tumour cells. Fluorescent antiserum against tumour particles reacted strongly with both normal tubular cells and carcinoma cells, and in addition gave a relatively weak staining reaction with glomeruli. This is in accordance with the lower degree of specificity of the tumour antiserum, as revealed in the complement fixation tests.

Some information could be obtained about the distribution of the kidney specific antiserum. It is contained in the whole tubular system of the nephron; the relative concentration is in the following order: proximal convoluted tubules, distal convoluted tubules, Henle loops, collecting tubules. This distribution over the whole nephron contrasts to the distribution of a number of enzyme systems investigated histochemically, most of which are confined only to certain parts of the nephron (Wachstein, 1955). As morphologically distinguishable parts of

the nephron are also functionally different, it seems impossible from these data to attribute to the kidney specific antigen a known physiological function.

Kidney is much less suitable than liver (Weiler, 1956) for identifying early effects of the carcinogen as quantitative differences in antigen content in certain foci; this is due to the rather inhomogeneous distribution histologically of the kidney specific antigen in normal kidney. Although the formation of stilboestrol induced kidney carcinomas is multicentric, and one would expect to find practically all stages of tumour formation in one section the earliest stages which could be identified using kidney specific antibody were small foci completely devoid of antigen, and having frequently yellow fluorescing granules. In haematoxylin-eosine-stained sections these foci appeared as accumulations of small basophilic cells.

The fluorescence microscopic appearance is different from that reported by Hill and Cruickshank (1953). These differences are easily explained by the different technique in preparing the fluorescent antisera : Hill and Cruickshank used whole kidney homogenate for immunisation, whereas in the present paper cytoplasmic particles were used.

SUMMARY

The antigenic relationship between normal kidney and stilboestrol induced kidney carcinoma have been studied, using fluorescein coupled antiserum globulin against cytoplasmic particles, and fresh frozen tissue sections.

Fluorescent kidney specific antibody does not give any staining reaction with tumour cells. This is in accordance with the preceding paper, where no kidney specific antigen could be found in tumour tissue.

In normal kidney tissue, the kidney specific antibody reacts with the cytoplasm of the tubular epithelium only. In the proximal convoluted tubules, there is a concentration of kidney specific antigen in the brush border.

Unabsorbed fluorescent kidney antiserum gives, in addition to the staining of normal tubular cells, a relatively weak staining with tumour cells.

Fluorescein-coupled antiserum against tumour particles strongly stains both normal epithelial cells and tumour cells, and gives a weak reaction with glomeruli.

I am much indebted to Professor A. Haddow for providing facilities at the Chester Beatty Institute, for helpful discussions, and for his interest in the work ; and to Dr. E. S. Horning for valuable discussion, and for providing the kidneys from hamsters treated with stilboestrol in his experiments.

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THE QUANTITATIVE TRANSPLANTATION OF SARCOMA 37 INTO SUBCUTANEOUS AIR POUCHES IN MICE

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In experiments reported previously (Hewitt, 1953*a*), it was found that when single-cell suspensions of Sarcoma 37 were injected into the subcutaneous tissue of adult mice, the proportion of injected sites in which a tumour subsequently grew was directly related to the number of cells injected. In a series of 16 " titrations " of S.37 cells, the number of cells required to give tumours in half the injected sites (the TD50) was calculated by the method of Reed and Muench (1938). A mean TD50 value of 1641 apparently viable sarcoma cells was obtained in the 16 experiments. When similar " titrations " were performed in new-born mice of the same strain, TD50 values of the order of 10 cells were obtained (Hewitt, 1953*b*). This difference was considered to be related to the smaller influence of immunogenetic factors in the new-born mice.

Two hypotheses were considered in an attempt to explain why the relative resistance of the mature mice could be overcome by the use of larger numbers of cells in the inocula.

Firstly, the resistance could be due to mutual interdependence among the injected cells, such that the chance of any one tumour cell escaping the resistance and proliferating to form a tumour was enhanced by its contiguity with other, similar cells. It is a common experience in tissue culture that a certain threshold number of starting cells is required in the culture if sustained growth is to be initiated. The fact that irradiated cells (devoid of proliferating power but with intact metabolism) will provide this " conditioning " factor (Puck and Marcus, 1955) shows that cellular metabolic products will enhance the proliferative power of intact cells of the same type.

An alternative explanation for the relatively high TD50 value using mature mice has reference to the heterogeneity of the cells of the inoculum. In this hypothesis, it is conceived that a small proportion of the cell population composing the inoculum are intrinsically peculiar in that they are resistant to the inhibitory factors in the adult host environment and are thus able to initiate the sustained growth required for the production of a palpable tumour. It is supposed that because these cells form only a small part of the injected population the TD50 is relatively high using adult mice.

If mutual contiguity of the injected tumour cells enhances the frequency of " takes " then a reduced frequency of takes should result in circumstances where the cells of the inoculum are dispersed more widely. It was previously believed that the dispersion of the injected cells could be progressively increased by increasing the volume of fluid in which they were injected. However, in a preliminary experiment in which the same number of cells was injected in inoculum volumes

varying from 0·2 to 2·0 ml., no significant variation of the frequency of " takes " was associated with variation of the volume of the inoculum. When a study was made of the distribution of suspensions (including cell suspensions) after injection into the subcutaneous tissue (Hewitt, 1954), it was found that the volume of the inoculum had no influence on the distribution of the particles. It was found that cells, for example, remained in a small zone near the point of the injecting needle while the fluid in which they were suspended diffused more widely. This process was such that if a number of cells were injected in a moderately large volume of fluid, they were in fact brought closer together during the process of injection. A wide distribution of injected cells was eventually effected by injecting cell suspensions into previously prepared air pouches in the subcutaneous tissue, and this paper reports an experiment in which the effect of dispersion of the inoculum on the frequency of takes was studied by this means. An additional application of the air pouch method—to study the influence of vascularity of the site of inoculation on the frequency of takes, is also described.

MATERIALS AND METHODS

Mice.—All mice used were from a colony of CBA mice maintained in this laboratory by brother-to-sister mating.

Tumour.—Sarcoma 37 was maintained in the ascitic form by serial intraperitoneal passage in albino mice. The methods of obtaining single-cell suspensions and of counting the viable cell density therein have been described previously (Hewitt, 1953*a*).

Production of subcutaneous air pouches.—Mice to receive air pouches were anaesthetised with ether and 4·0 ml. of air was injected into the dorsal subcutaneous tissue using a 5·0 ml. syringe carrying a Schick test needle. A horizontal fold of skin about 1 cm. from the base of the tail and in the mid-line was held up with forceps and the needle was inserted into the subcutaneous tissue in a cephalic direction, parallel to the vertebral column, until the point lay under the skin about 1 cm. in front of the raised fold of skin. The 4·0 ml. of air was then injected fairly slowly. If the air pouch tended to deviate from the mid-line, finger pressure was applied over the side to which deviation had occurred before injecting the remaining volume of air. By this means a symmetrically situated air pouch, as shown in Fig. 1, was quite simply produced. The form of pouches so made was studied by immersing anaesthetised mice bearing air pouches in a freezing mixture at −76° C., and making a mid-longitudinal bi-section of the frozen pouch with a razor. Fig. 2 shows a preparation of this kind in which the interior of the pouch has been revealed for inspection. The pouch was unilocular and ovoid in shape. The superficial surface was smoothly convex, and the deeper surface concave due to the protrusion into the pouch of the prominence of the vertebral column. Radio-opaque oil, after injection into the pouches of living mice, was seen radiologically to be distributed widely over the floor of the pouches. The pouches became gradually reduced in size after their production but were usually still appreciable by palpation after one week. Uncommonly, deflation occurred through the needle track immediately after injection of the air. This rarely happened, however, provided that a Schick test needle was used and that this was inserted at least 1 cm. under the skin before injection of the air. Free fluid was never seen in air pouches at any stage after their production. After injection of fluids into the air

pouches the mice were twice completely rotated to ensure a good initial distribution of the injected fluid.

Recording of tumour incidence.—Mice that had received injections of tumour cells were palpated every two or three days and the presence of tumours was recorded. As is usually found in the tumour-host system used, the smallest palpable mass almost invariably went on to form a visible tumour ; early regressions were rarely seen. Injected air pouches frequently gave rise to more than one tumour, whereas the growth of a tumour in an ordinary injection site could only be expressed as a single tumour. For this reason, the incidence of tumours was expressed in both cases as the proportion of sites growing tumours, whether these were multiple or not.

EXPERIMENTS AND RESULTS

(i) *Comparison of incidences of tumours after the injection of* 3000 *Sarcoma* 37 *cells into* (a) *the intact subcutaneous tissue,* (b) *subcutaneous air pouches.*

52 CBA male or female mice aged 350–450 days were distributed in two groups (C and A) such that each mouse of one group was paired with a mouse of a similar age and sex in the other group. All mice of Group C received 2 ordinary subcutaneous injections each of 0·2 ml. of a single-cell suspension containing a mean number of 3000 apparently viable Sarcoma 37 cells. One injection site lay between the shoulder blades in the mid line, the other being about 2 cm. posterior to this. All mice of Group A received a mid-dorsal air pouch into which 0·2 ml. of the same suspension as used for the Group C mice was injected. All injections were given under ether and mice of the two groups were injected alternately. All tumours appeared between the 10th and 20th days after injection but the non-tumour-bearing mice were observed for a further 30 days. The incidences of tumours in the various sites are given in Table I. One mouse of Group A died before a result was obtained. It will be seen from the table that there is no significant difference between the incidences in the various sites. Where multiple tumours appeared in the Group A mice, these were well separated, from which it may be assumed that a good dispersion of the cells occurred when they were injected into air pouches. From the results of this experiment it is concluded that the development of tumours from 3000 cells injected ordinarily into the subcutaneous tissue did not depend on their contiguity at the site of injection.

TABLE I.—*Incidence of Tumours After Injection of* 3000 *Sarcoma* 37 *Cells into* (a) *Intact Subcutaneous Tissue,* (b) *Subcutaneous Air Pouches.*

Site.	No. of sites injected.	No. of sites with tumours.	Incidence of tumours (per cent).	
Ordinary anterior	26	19	73	mean 67
Ordinary posterior	26	16	62	
Air pouch	25	17	68	

(ii) *Comparison of incidences of tumours after the injection of* 2500 *Sarcoma* 37 *cells into* (a) *untreated subcutaneous air pouches,* (b) *air pouches pre-treated with formic acid.*

From preliminary experiments it was found that the injection of 0·4 per cent formic acid solution into subcutaneous air pouches gave rise to hyperaemia in the wall of the pouch. This was clearly appreciable by the naked eye and was found

to be maximum on the third day after injection of the acid. At this time it was found that the pH of the wall of the pouch was normal, and it was assumed that no trace of the formic acid remained. One hundred male CBA mice aged 160–182 days were distributed equally into 2 groups (C and F). Each mouse of Group C was given a 4·0 ml. dorsal air pouch into which 0·2 ml. of normal saline was immediately injected. The mice were rotated to give a good initial distribution of the fluid. Mice of Group F were similarly treated except that 0·2 ml. of 0·4 per cent formic acid was injected into the pouches instead of saline. All injections were given under ether and mice of the two groups were injected alternately. On the third day after these injections, 0·2 ml. of a suspension of Sarcoma 37 cells containing an average of 2500 apparently viable tumour cells was injected into the pouch of each mouse of both groups, the mice being injected alternately. The mice were rotated as usual after the injections. All mice were subsequently palpated at 2–4 day intervals and the presence of tumours was recorded. More than one tumour frequently occurred in an air pouch, and where these were quite discrete they were recorded as separate tumours. Thirty-one days after injection all mice bearing tumours, many of which were now very large, were killed. The tumours were excised and weighed. All mice not yet bearing tumours were allowed to survive until the 58th day after injection, by which time only 4 of them had grown tumours. Two mice of Group C and 4 mice of Group F died during anaesthesia. In Table II are recorded, for the two groups: the proportion of injected mice bearing one or more tumours, the mean latent period between injection of the cells and the time a tumour first became palpable, and the mean weight of the tumours excised on the 31st day. None of the recorded differences are significant, from which it is concluded that the increased vascularity present in the formic acid-treated pouches at the time they were injected had no significant influence on the growth of the tumours. Of the 95 air pouches of both groups which were injected with 2500 sarcoma cells, 36 grew no tumour, 40 grew one tumour, 14 grew 2 tumours and 5 grew three tumours. These figures are consistent with the statistical chances of getting 0, 1, 2 and 3 "taking units" in successive samples (inocula) drawn from a suspension having a mean density of 1 "taking unit" per sample (inoculum). But the mean number of apparently viable Sarcoma 37 cells per inoculum volume was 2500 cells. Thus, the results of this experiment are compatible with the hypothesis that only 1 out of 2500 of the apparently viable Sarcoma 37 cells injected proliferate to form a tumour.

TABLE II.—*Incidence, Mean Latent Period and Mean Weight of Tumours arising in* (a) *Saline-Treated Air Pouches,* (b) *Formic Acid-treated Air Pouches, After Injection into the Pouches of* 2,500 *Sarcoma* 37 *Cells.*

Pre-treatment of pouches.	No. of pouches injected.	No. of pouches with one or more tumours.	Proportion of pouches growing tumours (per cent).	Mean latent period (days).	Mean weight of tumours (g.).
Saline	48	35	73	23·3	1·2
Formic acid	46	28	61	20·8	1·6

This experiment, with various modifications, was repeated. Again, no significant enhancement of the proportion of tumours grown in pouches resulted from pre-treatment of the pouches with formic acid.

DISCUSSION

The production of multiple tumours in separate situations after the injection of dilute sarcoma cell suspensions into air pouches confirms that this procedure produces a satisfactory dispersion of the injected cells. In spite of this wide separation of the injected cells, there was no reduction of the incidence of tumours from that given by similar inocula injected in the ordinary way, when the injected cells are confined within a very small locus (Hewitt, 1954). It is therefore concluded that the proliferative power of the injected sarcoma cells is not enhanced by their contiguity with one another at the site of implantation. In experiments described previously (Hewitt, 1953*a*) it was shown that the tumour incidence from small inocula of Sarcoma 37 cells was not affected by the simultaneous injection of a larger inoculum of similar cells at some other subcutaneous site.

The fact that sarcoma cells injected into air pouches proliferate as readily as those injected ordinarily is rather surprising when the many differences between these two environments are considered. The air pouch cells are initially in a heterogeneous environment with connective tissue on one side and moist air on the other ; before their invasion of the connective tissue, they are not subject to the tissue tension which is exerted round cells deposited in the tissue by ordinary injection ; they are adjacent to vascular tissue only on one side and their chances of embedding preferentially in the vicintiy of a capillary may be considered to be proportionately reduced as compared with cells that are initially surrounded on all sides by vascular connective tissue. None of these apparent disadvantages of the cells injected into air pouches had an appreciable effect on their capacity to proliferate into palpable tumours. Moreover, increasing the vascularity of the air pouches led to no enhancement of that capacity. These considerations encourage a conclusion that the local tissue environment encountered by the injected cells is optimal under a variety of conditions. It is permissible to make a similar conclusion from the ingenious experiments of Molomut, Spain, Kreisler and Warshaw (1955), who found that the frequency of takes and rate of growth of two mouse sarcomata were unaffected by the production of an allergic inflammatory response at the site of implantation. It appears that conditions at the site of implantation consititute less of a hazard to the implanted cells than it is often supposed.

The findings that have been described favour the hypothesis that was referred to in the introduction. This is, that the inoculum of tumour cells is a heterogeneous population of which only a small proportion of the cells possess intrinsic properties which enable them to proliferate in the subcutaneous tissue of the adult host. As explained previously, this proportion is estimated at about 1 : 2500 for the tumour-host system employed in the experiments described here. That the intrinsic peculiarity of the effective cells may be related to their chromosomal ploidy is suggested by the results of the immunoselection experiments of Hauschka, Kvedar, Grinnell and Amos (1956). On the other hand, it has been found that, in the case of Sarcoma 37, quantitative serial transplantation in a particular strain of mouse does not result in a progressive alteration of the number of cells required to give a constant incidence of tumours. It must therefore be assumed

EXPLANATION OF PLATE.

FIG. 1.—A dorsal subcutaneous air pouch in a CBA mouse.

FIG. 2.—CBA mouse with subcutaneous air pouch, part of the wall of which has been removed. The pouch is unilocular.

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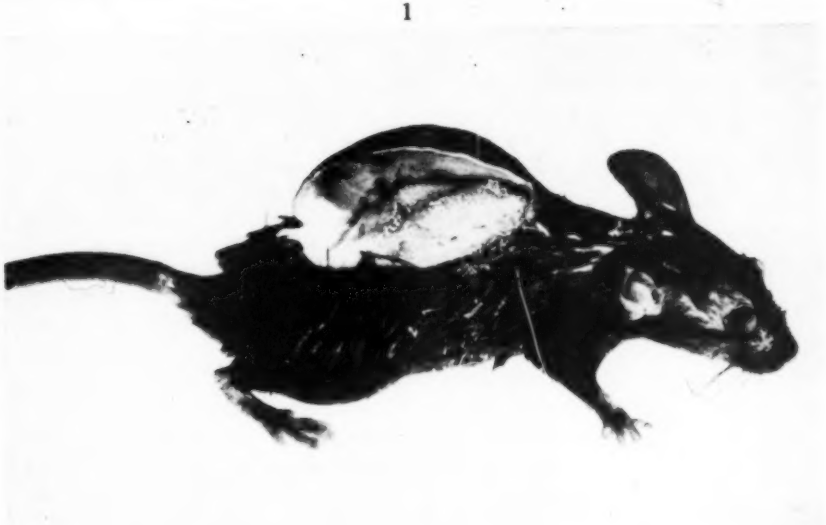

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Hewitt.

that a similar spectrum of cells arises in each tumour in the course of proliferation, although the property of transplantability is confined always to a small section of that spectrum.

The use of air pouches in transplantation studies may have applications other than those described here. In particular, the technique provides a convenient way of altering the implantation site before the cells are transplanted, as was done here using formic acid. Selye (1953) used air pouches in rats to study endocrine influences on inflammation. He found that the wall of the air pouch tended to react as a single structure, so that substances injected locally at the periphery of a pouch induced uniform changes in it. The opportunity which air pouches provide of getting multiple tumours from a single inoculum of tumour cells suggests their use for obtaining single-cell tumour clones without having to obtain inocula consisting of only one cell. If a single-cell suspension is diluted so that a given inoculum volume gives rise to tumours in only half the injected pouches, then the fact that several transplantable cells in an inoculum can express themselves as discrete tumours makes it improbable that any one of the tumours arose from more than one cell. As has already been stated, exudate has never been found in pouches—even after the injection of large doses of ascitic tumour cells, so that there appears to be no likelihood of the secondary implantation of exfoliated tumour cells.

SUMMARY

1. No significant difference was found in the incidence of tumours arising from a constant small inoculum of Sarcoma 37 cells injected (*a*) into the subcutaneous tissue, under conditions where the injected cells were deposited close together, and (*b*) into subcutaneous air pouches, in which the injected cells became widely dispersed.

2. A constant small inoculum of Sarcoma 37 cells was injected into (*a*) a series of untreated air pouches, and (*b*) a series of pouches that had been rendered hyperaemic by the previous injection of dilute formic acid solution. There was no significant difference between the two series in respect of the incidence of tumours, the mean latent period before they appeared, or their growth rate.

3. It is concluded that neither the density of the injected cells at the site of implantation nor the vascularity of the site of implantation influenced the capacity of injected cells to proliferate into palpable tumours. These findings are discussed in relation to the heterogeneity of the cells composing the tumour cell population.

4. Further applications of the air pouch technique to transplantation studies are suggested.

I am indebted to the British Empire Cancer Campaign for grants during tenure of which this work was preformed.

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THE EFFECT OF EXCESS DIETARY METHIONINE ON THE RATE OF GROWTH OF RD3 SARCOMA

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THE retardation of the growth of normal rats by excess methionine has been demonstrated by various workers (Pilsum and Berg, 1950; Graham, Hier, Waitkoff, Saper, Bibler and Pentz, 1950; Wretlind and Rose, 1950) during the last few years. The probable mechanism operating to produce this effect is suggested by the work of Wiseman (1955) who showed that the mono-amino-mono-carboxylic acids compete for the concentrating mechanism of the hamster small intestine and that methionine completely inhibits the active uptake of glycine, L-proline and L-histidine when present in equimolecular amounts. Methionine, therefore, probably produces its growth inhibiting effect by interfering with the active uptake of essential amino-acids by the normal tissues of the animal, and hence interferes with protein synthesis. However, this powerful inhibitory action of methionine on the amino-acid uptake by the intestine is less marked in the case of RD3 sarcoma cells (Wiseman and Ghadially, 1955), and although the degree to which amino-acids are concentrated by these cells is depressed there is not complete inhibition. It seemed likely, therefore, that if methionine is administered to rats bearing growing tumours it would either have little effect on the rate of tumour growth or it might even stimulate the tumour growth by making available amino-acids that would otherwise have been utilized by the normal tissues. The experiments described below were carried out to test this hypothesis. The results show that while the rate of growth of normal tissues is cut down, the rate of growth of the neoplasm is significantly increased.

METHODS

Tumour

The RD3 sarcoma used in these experiments was originally induced by 1 : 2 : 5 : 6-dibenzanthracene injection into the right flank of an inbred strain of albino rats and has been successfully transplanted subcutaneously in this strain over a period of 20 years.

All the animals in these experiments were inoculated subcutaneously in the right flank with 0·2 ml. of a thick pasty suspension of tumour mince. The use of a thick paste instead of a watery suspension afforded a fairly accurate and simple method of administering equal amounts of tumour material to a group of animals, as problems due to cells sedimenting out during the course of injection did not arise To each ml. of tumour paste was added approximately 100 mg. of streptomycin base and 50,000 units of crystalline penicillin, and the whole procedure was carried out with strict aseptic technique. There was never any macroscopic evidence of infection in any of the tumours induced by this method. Material obtained from

a single tumour was not adequate in quantity to inoculate all the animals used in an experiment and therefore two or three tumours were used. Care was taken to ensure that equal numbers of animals in each of the experimental and control group, or groups, received material from the same tumours. Thus, any difference arising from the different growth potentialities of different parent tumours used for transplanting were evenly distributed throughout the animal groups employed.

Diet

The basic diet used throughout these experiments was Diet 86, purchased from North-Eastern Agricultural Co-operative Society, Ltd., Bannermill Place, Aberdeen, and the theoretical composition is : soluble carbohydrate, 53·4 per cent; protein, 20·0 per cent.; fat, 3·8 per cent ; fibre, 3·3 per cent ; ash, 5·2 per cent ; moisture, 14·3 per cent. Enough food for all groups of animals was obtained in one batch at the start of the experiments, and the pellets were broken up in a mechanical crusher. Portions of this powdered food were enriched with 2 per cent or 4 per cent DL-methionine, as required, and then the enriched food powder and ordinary food powder were worked into stiff cakes by the addition of small quantities of water. The cakes were then made into pellets and dried in a fan-ventilated oven at 60° C. for 24 hours and subsequently stored in a dry cool place and used within a few days of manufacture.

Feeding technique

All animals were housed in separating cages and were weighed at the commencement of the experiment, and then re-weighed almost every day during the course of the experiment. The feeding régime was commenced on the same day as transplantation of the tumour. Group A animals received the 2 per cent or 4 per cent. DL-methionine-enriched food *ad lib*, while Group C received ordinary food *ad lib*. Group B were fed the ordinary food, but the amount that they received was such as to keep their total body weight the same as that of Group A. This required the daily weighing of the food given to each group and the weighing of the unconsumed food of the previous day. The amount of dietetic restriction imposed on Group B was estimated from their total weight and daily food consumption. Group B was only required when a diet enriched with 4 per cent DL-methionine was used.

RESULTS

Table I shows the effect on the rate of tumour growth of feeding a diet enriched with 4 per cent DL-methionine. As animals fed such a diet (Group A) ate considerably less than those on an ordinary diet (Group C) it was necessary to include a group of animals (Group B) whose mean body weight was kept equal to that in Group A. This was achieved by restricting the otherwise normal diet fed to Group B. It was found that the amount of food fed to Group B had to be appreciably less than that eaten by group A and the interpretation of this is discussed below. The mean body weights of the animals in all three groups were very nearly equal at the start of the experiment, and in groups A and B at the end of the experiment. The results indicate that methionine in this quantity stimulates the rate of tumour growth and it will be seen that the final tumour weight in Group A is significantly greater than in Group B. The final tumour weight in Group C is also greater than

that in Group B although the difference here is only on the borderline of significance. However, an experimental period of about twice this one would probably be necessary to show a marked difference in rate of tumour growth due to difference in body weight (Rous, 1914).

TABLE I.—*Effect of Methionine on Rate of Growth of RD3 Sarcoma. 4 per cent DL-methionine Added to Diet.*

(Group A animals fed methionine-enriched diet *ad lib.* Group B animals fed a restricted ordinary diet to keep their mean weight equal to that of Group A. Group C fed ordinary diet *ad lib.* Experiment started on same day as transplantation of tumour. Experimental period 12 days. Figures shown are mean for each group and standard deviation.)

	Initial body wt. (g.).	Final body wt. (excluding tumour wt.) (g.).	Final tumour wt. (g.).	Total food eaten per rat (g.).
Group A (28 rats)	236±26	208±21	14·5±7·1	161
Group B (31 rats)	238±28	200±22	9·9±5·7	123
Group C (25 rats)	242±22	247±34	13·1±7·1	332

The results in Table II show that the addition of 2 per cent DL-methionine to the ordinary diet is insufficient to produce any effect on the rate of tumour growth. As such a diet was eaten freely by the animals, and as the final body weights of Groups A and C were not significantly different, there was no need for a control Group B.

It should be noted that the rate of tumour growth in Group C in Table I was different from that in Group C in Table II. This is largely due to the differing rates of growth of transplanted tumours from different samples of tumour pastes, and hence the rates of growth of the transplanted tumours in the series in Table I should not be compared with the rates in the series in Table II.

TABLE II.—*Effect of Methionine on Rate of Growth of RD3 Sarcoma. 2 per cent DL-methionine Added to Diet.*

(Group A animals fed methionine-enriched diet *ad lib.* Group C animals fed ordinary diet *ad lib.* Experiment started on same day as transplantation of tumour. Experimental period 11 days. Figures shown are mean for each group and standard deviation.)

	Initial body wt. (g.).	Final body wt. (excluding tumour wt.) (g.).	Final tumour wt. (g.).	Total food eaten per rat (g.).
Group A (15 rats)	201±19	208±20	14·5±10·4	277
Group C (15 rats)	203±16	218±16	19·5± 9·7	295

DISCUSSION

In any experiments designed to reveal inhibition or stimulation of tumour growth the nutritional status of the animal is a factor of some importance. In a series of experiments Tannenbaum (1947) has shown that calorific restriction depresses or inhibits the genesis and growth of naturally-occurring and carcinogen-induced tumours, and Rous (1914) has shown that some transplanted tumours

are also adversely affected by dietetic restriction. It has been our experience that the rate of growth of the RD3 sarcoma is decreased in animals fed a restricted diet and losing weight. The weight of the experimental animal is actually of greater importance than its calorific intake, for if the basal metabolic rate is raised by administering thyroxine, and the body weight thereby caused to fall, there is a reduction of the tumour incidence corresponding to the fall in body weight even though the calorific intake has been increased (Tannenbaum and Silverstone, 1949). A study of the literature reveals that in such experiments tumour inhibition becomes demonstrable only when normal growth is retarded or when actual weight loss occurs. As we found that an experimental group of animals fed a diet enriched with 4 per cent DL-methionine consumed less than a group fed an ordinary diet *ab lib.* a group of animals on a restricted normal diet was included in that experimental series. The reason for the low consumption of the diet enriched with 4 per cent DL-methionine is not clear, but it seems likely to have been due to metabolic upset arising from the excess dietary methionine. When the difference in final body weight between Groups A and C is marked the rate of tumour growth in Group A must be compared with that of Group B for an evaluation of the effect of methionine, although Group C is of value in indicating the rate of growth on an ordinary diet fed *ad lib.* It will be noted that the amount of food consumed by Group A was less than that of Group B, although there is no significant difference between the body weights of these groups at the beginning or end of the experiment. Presumably this is due to the excess methionine in the diet of Group A which will decrease the active uptake of essential amino-acids by the normal tissues and hence protein syntheses. From the above results it can be seen that when 4 per cent DL-methionine was added to the diet there was a small but significant stimulation of the rate of tumour growth. It seems very unlikely that this greater rate of tumour growth was due to the adding of methionine to a diet inadequate in this amino-acid, as the body weight of the animals in Group A was equal to that of Group B even though the latter ate less. The addition of one missing essential amino-acid might reasonably be expected to show a difference in the carcass weight as well as in the weight of tumour tissue produced. A more likely explanation is that the excess methionine in Group A had a greater effect on the active uptake of amino-acids from the blood by normal tissues than it had on the active uptake of amino-acids by the sarcoma tissue. Such an explanation would fit in well with the findings of Wiseman (1955) and Wiseman and Ghadially (1955). The above experiments, therefore, suggest that when the diet of tumour-bearing animals is enriched with 4 per cent DL-methionine the tumour tissue is enabled to grow at a faster rate owing to the toxic effect of the excess methionine on the normal tissues, decreasing their ability to concentrate essential amino-acids from the blood, and thereby providing a readier source for the growing tumour.

SUMMARY

(1) It is shown that excess dietary methionine stimulates the rate of growth of RD3 sarcoma in the rat.

(2) The significance of this in conjunction with the inhibition of normal growth caused by excess methionine is discussed.

The authors are much indebted to Mrs. D. M. Tuck for her most valuable help during the experiments. They are also indebted to Professor H. N. Green

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TRACER STUDIES OF POTENTIAL RADIOSENSITIZING AGENTS. TETRASODIUM 2-^{14}C-METHYL-1 : 4-NAPHTHOHYDROQUINONE DIPHOSPHATE

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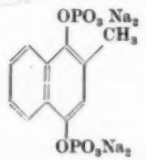
IF a non-toxic compound could be found which would concentrate selectively in tumour tissue and which would also increase the sensitivity of cells towards X-rays, it might be of use in the treatment of malignancy. This search for a radio-sensitizer has been the concern of these laboratories for a number of years, and initial studies have been mainly concerned with tetrasodium 2-methyl-1 : 4-naphthohydroquinone diphosphate (I).

The compound has several biological actions of interest. It is a synthetic Vitamin K substitute (Synkavit) by virtue of its ready dephosphorylation and oxidation to 2-methyl-1 : 4-naphthoquinone, but it also affects mitoses in cultures of chick fibroblasts (Mitchell and Simon-Reuss, 1947). It increases the antimitotic and chromosome fragmentation effects of X-irradiation on tissue cultures (Mitchell and Simon-Reuss, 1947, 1952a, 1952*b*) and also increases tumour retrogression by X-rays in rats bearing a Walker carcinoma (Mitchell, 1954). A small but useful increase in the survival time of patients undergoing X-ray therapy for inoperable carcinoma of the lung has also been reported (Mitchell, 1953). Furthermore, Mitchell (1954) has observed that after an injection of (I) into the rat, some internal organs, if dissected and treated with alkaline hydrogen peroxide, show a brilliant yellow fluorescence in ultra-violet light ; the fluorescence is especially strong on the growing edge of the tumour. It seemed likely that this indicated local accumulations of 2-methyl-1 : 4-naphthoquinone-2 : 3-oxide (II) derived from (I). Further information on possible selective concentration was therefore sought by studying the metabolism of (I) labelled with ^{14}C in the methyl group.



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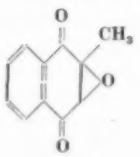

II

METHODS AND MATERIALS

Counting.—A windowless methane flow counter operating in the proportional region was used (Taylor and Sharpe, 1951).

Solutions for injection.—The compound (Andrews, Marrian and Maxwell, 1956), which was stored as the dry dibarium salt, was dissolved in a little water containing a trace of hydrochloric acid and converted to a solution of the tetrasodium salt

by percolating through a small column of Dowex 50 (sodium form). If not used at once, the solution was stored at 0° C. in a vial under nitrogen. The specific activity of the compound was determined both by plating out aliquots of known optical density ($\epsilon_M = 5600$ at 290mμ in 0·01 N hydrochloric acid), or, after dilution with carrier material, by wet oxidation to barium carbonate which was counted and a self absorption factor applied. Both methods gave an activity of $1{\cdot}83 \times 10^5$ cpm/μM. The radioactive purity of such solutions was determined before use by counting sections of a paper chromatogram of a drop of the solution run in saturated aqueous ammonium sulphate (80 vols.), 1M sodium acetate (20 vols.) and isopropanol (2 vols.). About 99 per cent of the activity was associated with the main ultraviolet absorbing spot (Rf 0·55) and the remainder with traces of slow moving material. Autoradiography of the dried paper strip and estimation of the amount of blackening of the film gave a similar result but was less convenient.

Animal experiments.—Laboratory stock albino male rats were used. The tumour bearing rats carried Walker 256 carcinoma used at the 2nd or 3rd passage from an ascites tumour. The tumour was implanted subcutaneously in the left flank some 10 days before the experiment and weighed 5–10 g. on the day of injection ; animals with small, rapidly growing tumours were selected. The compound (5–10 mg.) was injected into the tail vein and the animal immediately put into a perspex metabolism chamber connected when necessary to an apparatus for collecting respired carbon dioxide in carbonate-free alkali (Mackenzie, Chandler, Keller, Cross and du Vigneaud, 1949).

Preparation of the samples for analysis.—The animals were killed (chloroform) in a closed chamber which was later rinsed with water, and the water added to the urine sample. Immediately the animal was dead, blood was removed by cardiac puncture, the internal organs dissected, placed in weighed specimen tubes, and frozen in acetone/CO_2. The radioactivity in the carcass was determined by homogenising the frozen carcass in 2N sodium hydroxide ; aliquots of each layer of the centrifuged homogenate were oxidised to barium carbonate. The remainder of the solid material from the homogenate was dried and defatted by washing with methanol and refluxing with methanol/chloroform. If not analysed at once, the samples were kept at $-$ 20°. Storage at this temperature for 3 weeks did not affect the activity of the derived barium carbonate.

Analysis of tissues and blood samples.—Duplicate weighed samples (about 100 mg.) were quantitatively oxidised by the van Slyke-Folch reagent (Van Slyke, 1954) and the carbon dioxide isolated as barium carbonate under strictly standardised conditions (Wick, Barnet and Ackerman, 1949). The whole of the barium carbonate was plated out on an aluminium planchette, dried, weighed and counted to a standard error of less than 5 per cent. The activites of duplicate samples were within 10 per cent of the mean value.

Investigation of the urine.—The total activity in the urine was determined by plating out aliquots in infinitely thin layers. Two paper chromatographic separations were run, the solvents being (a) the one referred to above and (b) n-butanol (3 vols.) and methanol (1 vol.) saturated with water containing a few drops of acetic acid.

RESULTS AND DISCUSSION

Previous studies of the metabolism of tetrasodium 2-methyl-1 : 4-naphthohydroquinone diphosphate have given confused results. The fate, in rats, of

intramuscular injections of the compound labelled with ^{32}P has been studied by Neukomm (1954) and his colleagues (Neukomm, Peguiron, Lerch and Richard, 1953) who showed that exchange occurred between the compound and the inorganic phosphate pool. By extrapolation of their time curves, they deduced a curve denoting fixation and excretion of the compound and showed that the metabolism of the compound and of inorganic phosphate followed different and distinguishable courses ; fixation of the compound occurred mainly in metabolically active organs and in those concerned with storage, detoxification and excretion. However, Morrison and Crowley (1952) using the same label, concluded that the phosphate groups were quickly split off and that the resulting activity was distributed as inorganic phosphate. Since injected 2-^{14}C-methyl-1 : 4-naphthoquinone can be excreted as the hydroquinone diphosphate (Jaques, Millar and Spinks, 1954), injection of the phosphorus labelled hydroquinone diphosphate can quickly give rise to unlabelled 2-methyl-1 : 4-naphthoquinone which could then be reduced and phosphorylated by the phosphate pool to give the original injected compound with a much lower specific activity. These complications do not arise with the ^{14}C labelled compound, and the fixation and excretion of tetrasodium 2-^{14}C-methyl-1 : 4-naphthohydroquinone diphosphate by the rat is shown in Table I. The amount associated with the blood was estimated by oxidising a known volume to carbon dioxide and assuming that the total blood volume was 6·7 ml. per 100 g. body weight (Gartland and Koch, 1928). Only about 70 per cent of the injected radioactivity was recovered, and since the oxidation technique

TABLE I.—*Recovery of radioactivity from a rat* (237 *g.*) *injected intravenously* (*tail*) *with* 5 *mg. of tetrasodium* 2-^{14}C-*methyl*-1 : 4-*naphthohydroquinone diphosphate and killed* 1½ *hours later. Total injected activity* 2·66 × 10^6 *cpm.*

Tissue.	Total activity cpm. × 10^5.	Percentage of injected dose.
Urine	8·67	32·6
Blood	0·65	2·44
Organs	0·80	3·0
CO_2	0·26	1·0
Faeces	0	0
Carcass homogenised in aq. NaOH :		
(i) Solid	3·92	14·8
(ii) Liquid	4·3	16·2
Totals	18·6 × 10^5	70%

The solid fraction of the carcass homogenate was dried and defatted and then contained a total of 2 × 10^4 cpm. (about 1 per cent of the dose).

was better than 95 per cent effective, there remains about 25 per cent of the dose unaccounted for. Efficient homogenising and sampling of the carcass was not easy, and this may account for some of the loss. However, the recovery compares well with the similar experiment using 2-^{14}C-methyl-1 : 4-naphthoquinone where only 50 per cent of the activity was accounted for (Jaques, Millar and Spinks, 1954). The occurrence of activity in the respired carbon dioxide is of interest since it has been reported (Solvonuk, Jaques, Leddy, Trevoy and Spinks, 1952) that 2-^{14}C-methyl-1 : 4-naphthoquinone was not oxidised to carbon dioxide by the mouse ; the low specific activity of the starting material available at that time may explain this discrepancy. To confirm this oxidation, a 24 hour investiga-

tion of respiratory carbon dioxide was carried out after a tumour bearing rat had been given an intravenous injection of 8·5 mg. (I). The results are shown in Fig. 1 while Fig. 2 shows the total activity respired by the rat over the 24 hour period. It can be seen (Fig. 1) that there is a maximum both in the specific activity and of the rate of respiration of carbon dioxide at about 2 hours after injection, after which both values fall, then remain constant for the rest of the experimental period—the respired activity being about 0·25 per cent of the dose

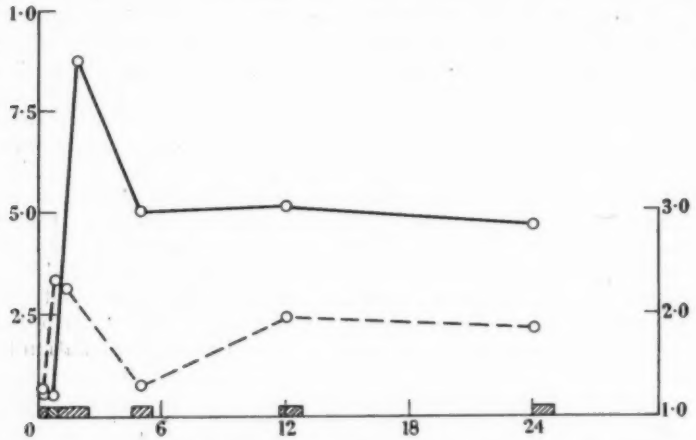

FIG. 1.

L.H. Ordinate : cpm./gm. $BaCO_3 \times 10^3$.
R.H. Ordinate : CO_2 expiration rate in gm. $BaCO_3$/hr.
Abcissa : Time in hours after injection.
Radioactivity in CO_2 respired by the rat following an injection of 8·5 mg. tetrasodium 2-^{14}C-methyl-1 : 4-naphthohydroquinone diphosphate.
Full curve : specific activity of respired CO_2.
Broken curve : rate of respiration of CO_2.
Shaded areas denote periods when CO_2 was collected.

per hour. These maxima could infer an initial period of high intracellular concentration in the tissue and would suggest that localisation of the compound did occur to some extent. The later, steady respiration of activity shows that 2-^{14}C-methyl-1 : 4-naphthoquinone is also broken down to carbon dioxide.

The products excreted in the urine by the rat were investigated by paper chromatography ; results agreed with those previously reported for the metabolism of 2-^{14}C-methyl-1 : 4-naphthoquinone (Jaques, Millar and Spinks, 1954). The methanol/butanol solvent separated 3 active components from rat urine, one having the position normally occupied by the injected compound (I). The other two (Components A and B) corresponded with the glucuronide and sulphate already described (Fig. 3 and Table II). (Jaques, Millar and Spinks; 1954 ; Rickert, 1944, 1951 ; Doisy, 1949). The ammonium sulphate system also indicated the presence of the injected material but did not separate the other active components. The excretion of (I) even 24 hours after injection did not, of course, indicate that the compound had not undergone rapid dephosphorylation.

TABLE II.—*Percentage of the Total Radioactivity in rat urine at intervals after intravenous injection of 5 mg. (I). The components refer to the active peaks in Fig. 3.*

Fraction.	Injected Compound (I).	Component A.	Component B.
0–2 hours	9·9	63	23·0
2–6 hours	5·6	71	23·8
6–24 hours	4·4	72	23·0

The distribution of the intravenously injected compound in the various organs of the tumour bearing rat is shown in Table III in terms of the Differential

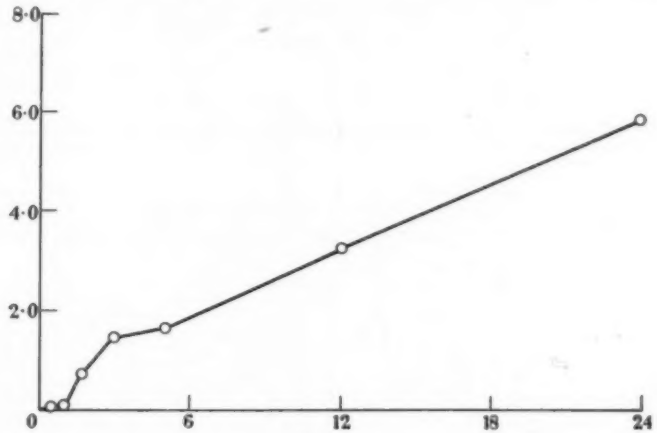

FIG. 2.

Ordinate: Percentage of injected radioactivity in respired CO_2 following injection of 5 mg. tetrasodium-2-^{14}C-methyl-1 : 4-naphthohydroquinone diphosphate.
Abcissa: Time in hours after injection.

Absorption Ratio (cf. Moore, Tobin and Aub, 1943) (D.A.R.), a convenient means of studying possible localisation of a compound in a tissue. The D.A.R. is defined as

$$\frac{\text{Observed activity/g. of tissue}}{\text{Total injected activity/g. of total rat weight.}}$$

Thus, if a compound were uniformly distributed throughout the body and not excreted, the D.A.R. of each tissue would be 1·0. Since excretion usually occurs, the D.A.R. decreases with time. Moreover, since the technique of wet oxidation was almost quantitative, the D.A.R. can also be calculated in terms of dry tissue thus

$$\text{D.A.R. (dry tissue)} = \frac{\text{Activity/g. } BaCO_3 \text{ from the tissue}}{\text{Total injected activity/g. } BaCO_3 \text{ from the whole rat}}$$

The mean $BaCO_3$ equivalent of the whole rat was taken to be the same as for muscle. These results are given in Table IV. Tables II, III and IV show that the

compound concentrates in the organs concerned with Vitamin K function and with detoxification and excretion. But, on a dry weight basis (Table IV) the D.A.R. of tumour is several times higher than that of muscle, especially for short periods after injection.

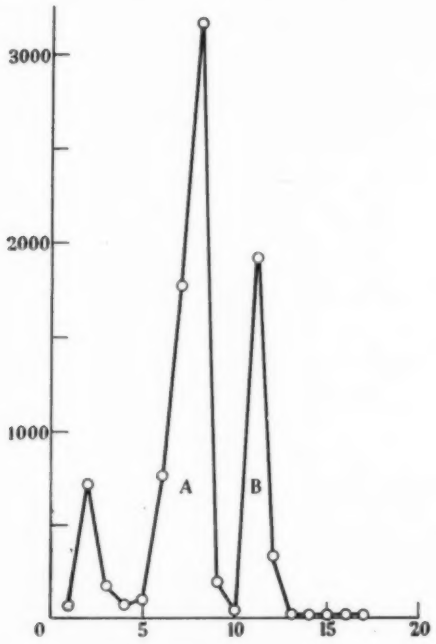

FIG. 3.

Ordinate: Radioactivity in cpm.
Abcissa: Fraction number: (each fraction represents 2 cm. of the chromatogram).
Distribution of radioactivity along a chromatogram of urine excreted by a rat bearing a Walker carcinoma during 2 hours following an injection of 5 mg. tetrasodium 2-^{14}C-methyl-1 : 4-naphthohydroquinone diphosphate (Butanol/methanol system). The three peaks of activity are, respectively, the injected compound (I), and two metabolites A and B (see Table II).

It is probable that the figure found for the D.A.R. of the tumour is minimal since the whole tumour tissue contains more stroma than tumour cells. Since fluorescence studies indicated that nearly all the fluorescence arose from the tumour cells and not from the stroma (Mitchell, 1954), this suggests that the actual D.A.R. in the tumour cells must be much higher than that measured for the tumour tissue as a whole.

These results add force to earlier indications that 2-^{14}C-methyl-1 : 4-naphthohydroquinone bis (disodium phosphate) can concentrate in tumour tissue for short periods and give hope that a compound may be developed with a similar but more pronounced radiosensitizing effect possibly acting by virtue of selective concentration in tumour tissue.

TABLE III.—*The ^{14}C Content of Tissue Samples from Rats with The Walker 256 Carcinoma following Intravenous Injection of 5 to 10 mg. of ^{14}C-Compound I.*

	D.A.R. (wet tissue).				
	1 hour.		1½ hours.	2 hours.	24 hours.
Rat No.	14/1	21/1	17/1	18/1	1/2
Tumour	0·3	0·24	0·21	0·095	0·12
Muscle	0·15	0·11	0·05	0·093	0·10
Testis	0·26	0·23	0·16	0·13	0·17
Kidney	2·0	2·1	1·1	0·67	0·46
Spleen	0·3	0·24	0·16	0·12	0·11
Liver	0·66	0·61	0·28	0·29	0·18
Blood	0·67 (4·4%)	0·53 (3·6%)	0·37 (2·4%)	0·27 (1·8%)	0·12 (0·79%)
Urine	—	—	32%	—	22%
Faeces	—	—	—	—	5·3%
CO_2	0·01%	—	—	0·03%	5·9%

Percentages in the lower half of the table indicate percentage of the injected dose associated with the blood and excreta.

TABLE IV.—*The ^{14}C Content of Tissue Samples from Rats with The Walker 256 Carcinoma following Intravenous Injection of 5 to 10 mg. of ^{14}C-Compound I.*

	D.A.R. (dry tissue).				
	1 hour.		1½ hours.	2 hours.	24 hours.
Tumour	0·44	0·37	0·36	0·11	0·18
Muscle	0·13	0·10	0·06	0·08	0·16
Testis	0·43	0·42	0·27	0·23	0·28
Kidney	1·7	1·9	1·3	0·65	0·42
Spleen	0·31	0·22	0·18	0·13	0·12
Liver	0·58	0·45	0·26	0·23	0·17

SUMMARY

The metabolism of tetrasodium 2-^{14}C-methyl-1 : 4-naphthohydroquinone diphosphate has been studied in rats. About 30 per cent of the activity is excreted in the urine within 90 minutes of injection and activity is also found in respired carbon dioxide.

Measurement of the Differential Absorption Ratio of various internal organs shows that, for short periods after injection, the compound concentrates more in tumour tissue than in muscle, but that it also accumulates in organs concerned with detoxification, excretion and Vitamin K function.

The authors record their thanks to Professor J. S. Mitchell, F.R.S., for his continual interest and encouragement, and to Mr. E. A. King for his help with the animal experiments. One of us (D. R. M.) is indebted to the Medical Research Council for a research grant.

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THE INTRACELLULAR METABOLISM OF 3 : 4 BENZPYRENE. THE ROLE OF ENZYMES IN THE FORMATION OF BENZPYRENE METABOLITES BY LIVER COMPONENTS

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EARLIER studies (Calcutt and Payne, 1954*a*, 1954*b*, 1954*c*) have shown that benzpyrene is metabolised by both the particulate and soluble components of rat and mouse liver tissue. On the basis of this widespread distribution of intracellular sites of oxidation it was suggested that the process was non-enzymatic. Previously, the same suggestion was made by Casu and his associates (Casu *et al.*, 1951) as a result of their studies on the formation of benzypyrene metabolites by autoclaved liver homogenates.

Casu and his asssociates used homogenates of whole liver and filtrates—roughly representing the soluble tissue fractions—in their experiments. In view of the later findings by Calcutt and Payne (1954*a*, 1954*b*, 1954*c*) that liver nuclei, mitochondria, microsomes and three separate fractions of the supernatant will all cause the oxidation of 3 : 4 benzpyrene it has been considered advisable to investigate whether the mechanism is similar in all cellular substrates.

EXPERIMENTAL

The animal material used in these experiments was derived from the livers of freshly killed rats or mice. The livers were homogenized and separated into components as described previously (Calcutt and Payne, 1954*a*, 1954*b*). The benzpyrene was used as a colloidal suspension in distilled water. Examinations for the detection of benzpyrene metabolites were those used previously, the findings being expressed in the terminology devised by Weigert and Mottram (1946). These authors isolated and characterized two components as primary metabolites and labelled them as BpX_1 and BpX_2. By physical methods and analogy with known derivatives of other polycyclic hydrocarbons these were shown to be :

$8(OR_1)$-9(OH)-8-9, dihydro-3 : 4 benzpyrene—BpX_1 and $8(OR_1)$-$9(OR_2)$-8, 9 dihydro-3 : 4 benzpyrene—BpX_2. The radicals R_1 and R_2 are unknown. A further compound was labelled BpF_1 and believed to be $8(OR_1)$-3 : 4 benzpyrene. This occurred as an intermediate in the breakdown of BpX_1 to 8(OH) 3 : 4 benzpyrene, and this last compound was designated for convenience as BpF_2.

First series of experiments

Homogenates of mouse or rat liver were prepared in Tyrode solution and separated by centrifugation into nuclei, mitochondria, microsomes, top, middle and bottom supernatant fractions. Each fraction was maintained at 100° C for

one hour. After cooling benzpyrene colloid was added to each fraction, the whole thoroughly mixed and placed in an incubator at 37° C in total darkness. This last precaution was taken in order to obviate any effects arising from the photosensitizing action of the benzpyrene. At varying time intervals the fractions were removed and examined for the formation of benzpyrene derivatives.

Throughout the experiments a check was kept on the course of events by examination of samples under a U.V. lamp fitted with a Woods glass filter. The appearance of the samples is described in Table I. Immediately after the addition of the benzpyrene colloid any other fluorescence was swamped by the intense yellow-green of the hydrocarbon particles.

TABLE I.—*Appearances of Fractions under U.V. Illumination.*

		Nuclei.	Mitochondria.	Microsomes.	Top supernatant.	Middle supernatant.	Bottom supernatant.
Mouse liver fractions	As extracted	Dull blue	Pale blue	Blue-violet	Blue	Blue-green	Green
	After heating	Blue	Dull blue in green liquid	Yellow	Blue-green	Blue-green	Blue-green
	After incubation	Bright blue	Blue-white	Blue-white	Pale blue	Pale blue	Pale blue
Rat liver fractions	As extracted	Dull blue	Pale blue	Pale blue	Blue-green	Green	Green
	After heating	Blue	Violet in yellow liquid	Yellow	Blue-green	Blue-green	Blue-green
	After incubation	Blue-white	Blue-white	Blue-white	Pale blue	Pale blue	Pale blue

The results obtained from the examination for benzpyrene derivatives are given in Table II. These results are not very convincing, since even where positive findings were made the amounts of derivatives were small as compared with those obtained under similar conditions from unheated fractions. A possible explanation was the fact that when the fractions were heated the protein content settled out in the tubes as a hard tarry mass. This could only be broken with difficulty and obviously much of it never came into contact with the benzpyrene particles. To overcome this problem a further run of experiments was undertaken.

TABLE II.—*Benzpyrene Metabolites After Incubation of Heated Liver Fractions with Benzpyrene*

	Period of incubation.	Nuclei.	Mitochondria.	Microsomes.	Top supernatant.	Middle supernatant.	Bottom supernatant.
Mouse liver fractions	16 hours	*Nil*	*Nil*	*Nil*	*Nil*	BpX_2	*Nil*
	8 days	*Nil*	? BpX_2	*Nil*	BpX_2	BpX_2	BpX_1
Rat liver fractions	22 hours	*Nil*	*Nil*	*Nil*	BpX_2	BpX_2 BpX_1	BpX_1
	21 days	*Nil*	*Nil*	? BpX_2	? BpX_2	BpX_1	BpX_1 BpF_1

Unchanged hydrocarbon was present in all fractions. *Nil* indicates nothing found. ?, amount too small for positive identification.

Second series of experiments

Liver fractions were prepared as previously and then transferred to 70 per cent glycerol. In the case of the liquid supernatant fractions this was achieved by the addition of sufficient pure glycerol to give a final concentration of 70 per cent. The fractions were then maintained at 100° C for 1 hour as previously. Under these conditions the coagulated proteins formed a light fluffy mass and remained in suspension. The benzpyrene was added as a colloid as previously, but this time went partly into solution in the glycerol. Incubation and examination for benzpyrene derivatives were as in the first series of experiments. The findings are tabulated in Table III. The course of events could not be followed under the U.V. lamp in this series as the glycerol has a dull blue fluorescence of its own.

TABLE III.—*Benzpyrene Metabolites After Incubation with Benzpyrene of Liver Fractions Heated in 70 per cent Glycerol.*

	Period of incubation.	Nuclei.	Mitochondria.	Microsomes.	Top supernatant.	Middle supernatant.	Bottom supernatant.
Mouse liver fractions	17 hours	BpX_2	BpX_2	BpX_2	BpX_2	BpX_2	BpX_1
	32 days	BpX_2	BpX_2	BpX_2	BpX_2	BpX_2	BpX_1
						BpX_1	BpF_1
Rat liver fractions	16 hours	BpX_2	BpX_2	BpX_2	BpX_2	BpX_2	BpX_1
						BpX_1	BpF_1
	7 days	BpX_2	BpX_2	BpX_2	BpX_2	BpX_2	BpX_1
						BpX_1	

In this series metabolites were found in all fractions and corresponded with those found in earlier work using fresh material.

In all experiments the tubes for incubation received an addition of 50,000 units of soluble penicillin, this being found effective in preventing the growth of bacteria. Control incubation of penicillin with benzyprene gave no indications of the formation of any hydrocarbon derivatives.

DISCUSSION

The results obtained are very suggestive of the formation of benzpyrene metabolites being a non-enzymatic process in all the liver sub-fractions. The technique of using heat as a method for the destruction of enzymes is, however, not completely satisfactory since some enzymes—notably, ribonuclease—are thermostable. Under the circumstances the case cannot be considered as proved as the actual cellular substrates involved in the oxidation processes are still unknown. Alternate supporting evidence for the purely chemical basis of the oxidation of the benzpyrene derives from the widespread sites of the oxidation as compared with the localization of most enzyme systems, and the fact that compounds apparently identical with benzpyrene metabolites have been obtained by Calcutt (1949, 1950) and Garzia and Dansi (1953) using simple chemical systems.

Even if it be accepted that benzpyrene is metabolised in the animal body by essentially non-enzymatic means this does not exclude effects upon enzyme systems. It has been shown that benzpyrene and other carcinogenic hydrocarbons cause inhibition of proteolytic enzymes (Rondoni, 1955), whilst the enzymes of isolated mitochondria are activated or inhibited according to the

hydrocarbon dosage applied (Dianzani, 1953). It seems possible, then, that whilst metabolism of benzpyrene may be non-enzymatic the enzyme systems of the tissues in which metabolism occurs may also be affected.

The suggestion that benzpyrene may be oxidized by purely chemical means raises another important issue. In the case of the related non-carcinogens, naphthalene, anthracene and phenanthrene it has generally been assumed that metabolism is enzymatic. In fact, Pullman and Pullman (1955) have postulated the intervention of an enzyme system in the metabolism of the smaller hydrocarbons in order to offer a logical explanation of the experimental findings. The suggested non-enzymatic metabolism of benzpyrene now leads to the proposal that the carcinogenic and non-carcinogenic hydrocarbons are metabolized in different fashions.

The non carcinogenic hydrocarbons have been found to be excreted as diols or derivatives conjugated as mercapturates or glucuronides whilst the only identified products of the carcinogenic homologues have been phenols and quinones. It has been shown by Gutman and Wood (1950*a*, 1950*b*) that benzpyrene is not excreted as a mercapturate, whilst no evidence for any excretion of conjugated derivatives of dibenzanthracene could be obtained (Dobriner, Rhoads and Lavin, 1942). Thus such little experimental evidence as is at present available is in keeping with the proposed distinction in metabolism between carcinogenic and non-carcinogenic hydrocarbons. Further evidence is essential and may well assist to elucidate the problems of differences of biological behaviour of the different polycyclic hydrocarbons.

SUMMARY

Rat and mouse livers have been separated centrifugally into nuclei, mitochondria, microsomes and top, middle and bottom supernatant fractions. After an assumed destruction of enzymes by holding these fractions at 100° C. for one hour they were found to metabolize benzpyrene in the same manner as fresh liver fractions. It is suggested that the metabolism of benzpyrene is essentially a non-enzymatic process.

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"FREE" ENZYMIC ACTIVITY OF MITOCHONDRIAL FRACTIONS FROM LIVER TUMOURS

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As first shown by Kielley and Kielley (1951), mitochondria freshly isolated from liver show little adenosinetriphosphatase (ATP-ase) zactivity, but "latent" activity becomes manifest when the preparations are subjected to procedures such as freezing or dilution with a hypotonic medium. Accordingly, it is generally considered that a low level of "free" ATP-ase activity signifies that the mitochondria have been isolated in an essentially intact state. The situation is similar in the case of acid phosphatase (Berthet and Duve, 1951).

Homogenates of primary liver tumours, and mitochondrial fractions derived therefrom, have been extensively studied with respect to enzyme levels in comparison with those in normal liver (Allard, 1955). Such studies have not, however, included measurements of "free" activity as distinct from "total" activity. This aspect has now been studied, with the possibilities in mind that tumour mitochondria may have impaired integrity *in vivo* or may be so fragile as to be readily damaged during their isolation. Particular care has been taken to ensure that the "liver tumours" studied included some hepatomas which, unlike mixed tumours, could reasonably be compared with normal liver.

EXPERIMENTAL

Most of the experiments were performed with albino male rats, of the Institute colony, which had been maintained for 12–14 weeks on a semi-synthetic diet containing 0·06 per cent 3′-methyl-4-dimethylaminoazobenzene as in the experiments of Griffin, Nye, Noda and Luck (1948); these rats were killed 16–18 weeks from the initiation of azo-dye feeding. The rats used for the study of "pre-cancerous liver" were killed after only 4 weeks of azo-dye feeding. A few initial experiments were performed with male rats of the albino strain or of the "August" strain, which had been maintained for 9 months on a "20 per cent protein diet" (Elson, 1952) containing 0·075 per cent *p*-dimethylaminophenylazo-2-naphthalene (Mulay and Congden, 1953); however, the number of tumours found at autopsy (10–12 months from the initiation of azo-dye feeding) was fewer than had been anticipated. Since the enzyme determinations gave no indication of a difference between rats thus treated and rats fed 3′-methyl-4-dimethylaminoazobenzene, the values were combined for the purposes of calculation.

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The data for each set of experimental rats were compared with those for control rats which had been maintained on the same diet (without carcinogen) and which were studied simultaneously. The rats were finally killed with "Nembutal" and the livers removed, one or more tissue samples being taken from each liver. The samples were homogenized in 0·25 M sucrose solution, which substantially maintains the "latency" of ATP-ase (Kielley and Kielley, 1951) and of acid phosphatase (Berthet and Duve, 1951). In a few experiments, however, the sucrose medium was replaced by a dextran-containing medium (Birbeck and Reid, 1956) which is more effective in preserving mitochondrial structure during isolation; the enzymic changes observed with tumour fractions isolated in this medium did not differ from those observed with the sucrose medium.

The procedures for the centrifugation of the nuclear and mitochondrial fractions were as described elsewhere (Reid, 1955), the latter fraction being sedimented at 12,000 *g* (15 minutes; 0°) and washed once without removal of the "fluffy layer". The washed fractions were resuspended in the sucrose medium and assayed after storage for two hours at 5°. Assays were also performed on some "supernatant" fractions obtained by a further high-speed centrifugation (20,000 *g*, 90 minutes) of the material which did not sediment at 12,000 *g*.

ATP-ase was determined at pH 7·4 in the presence of Mg^{++} (0·002 M), essentially as described by Lardy and Wellman (1953). Incubation was carried out at 30°, for 15 minutes with shaking, in the presence of 0·003 M ATP (disodium salt, "99 per cent ATP", as suppplied by Nutritional Biochemicals Corporation). The supernatant fluid obtained by centrifugation after addition of trichloroacetic acid was analyzed for orthophosphate, the amount of which is a valid measure of ATP-ase activity (Novikoff, Hecht, Podber and Ryan, 1952; Kielley and Kielley, 1953).

Acid phosphatase was determined at pH 5·0 (0·1 M acetate buffer) in the presence of sodium β-glycerophosphate, essentially as described by Berthet and Duve (1951); incubation was carried at 37° for 10 minutes ("free" activity) or for 20 minutes (total activity). Orthophosphate was finally determined as in the ATP-ase assays, the amount found in the assays of "free" activity being doubled so that all the data (cf. Fig. 2) refer to an incubation time of 20 minutes.

For the determination of "free" activity in these assays, freshly isolated mitochondrial fractions were used, and 0·25 M sucrose solution was used as diluent in the assays. For the determination of "total" activity, the tissue samples were diluted with water, and frozen and thawed at least once (ATP-ase) or at least 8 times (acid phosphatase), so as to liberate the "latent" activity.

Nitrogen was determined by the micro-Kjeldahl procedure, the ammonia being determined in Conway units.

RESULTS

The tissue studied in some experiments was obtained from rats which had been given azo-dye for a short period as indicated above; this tissue was designated *pre-cancerous liver*. The tissue samples from rats which had been given azo-dye for a prolonged period were sub-divided, for the purpose of assessing the biochemical findings, into the following categories on the basis of the histological description:— *liver tissue without tumours, mixed hepatoma and cholangioma, hepatoma with little necrosis* and *hepatoma with marked necrosis*. The "liver tissue without tumours"

showed the same histological changes (Edwards and White, 1941; Opie, 1944; Orr and Price, 1948; Hartroft, 1954) as the pre-cancerous liver, but differed from the latter in the longer duration of azo-dye feeding; with further prolongation of this feeding, tumours would probably have developed.

All the rats which had been given azo-dye showed enlarged livers, the average weight (relative to body weight) being 3·0 per cent for control livers, 4·4 per cent for "pre-cancerous livers", and 5·6 per cent for the livers of rats given prolonged azo-dye treatment. Accordingly, a decrease in enzyme concentration (per mg. of fresh liver tissue, as in Fig. 1 and 2) does not necessarily signify that the total amount of that enzyme in the liver was decreased.

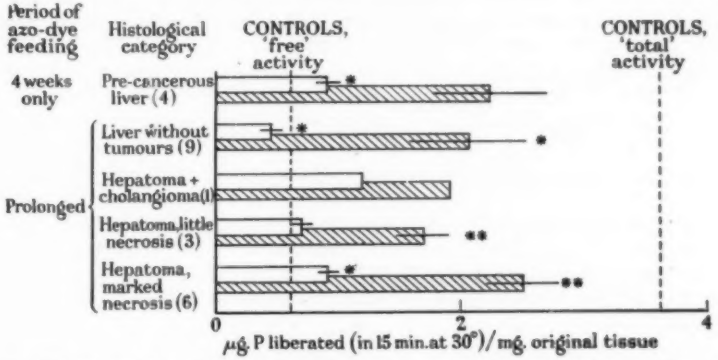

FIG. 1.—ATP-ase activity of mitochondrial fractions. In Fig. 1 and 2 unhatched bars represent "free" activity and hatched bars represent "total" activity. In Fig. 1–3 the standard error of the mean difference between each experimental value and the corresponding control value is indicated by a line, and the number of degrees of freedom is given parenthetically; for significant differences, the probability that the difference could be due to chance is indicated by * ($P < 5\%$), ** ($P < 1\%$) or *** ($P < 0{\cdot}1\%$).

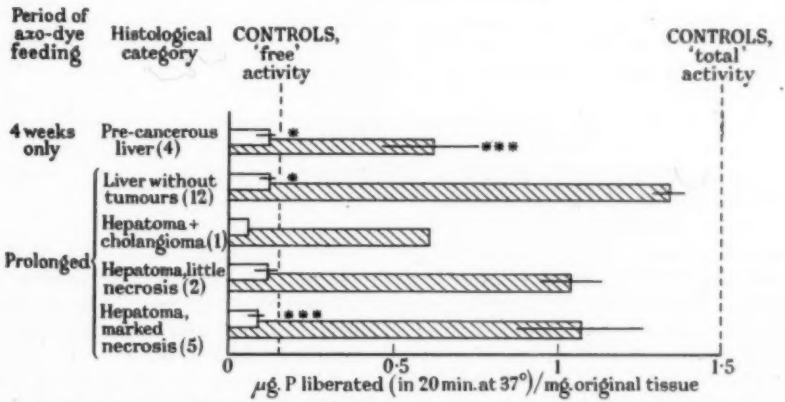

FIG. 2.—Acid-phosphatase activity of mitochondrial fractions. See legend to Fig. 1 for method of representing data.

As is shown in Fig. 1, the azo-dye feeding tended to diminish " total " ATP-ase activity, irrespective of the type of tissue studied. " Free " ATP-ase activity did not, however, decrease proportionately; indeed, this activity significantly exceeded that of the controls in the case of pre-cancerous liver and of hepatomas with necrosis.

The assays for " total " acid-phosphatase activity (Fig. 2) showed a marked fall in the case of pre-cancerous liver. With the other tissue samples this activity was not significantly decreased. The " free " activity tended to decrease with each type of tissue, although in the case of pre-cancerous liver the decrease was proportionately less than the decrease in " total " activity.

It was of interest to know if the decreases in " total " enzymic activity were a reflection of loss of mitochondrial material after azo-dye feeding, as previously

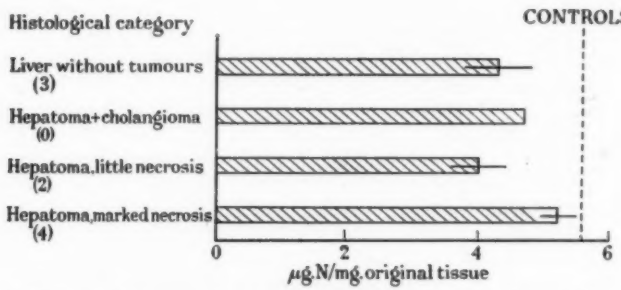

FIG. 3.—Amount of nitrogen in mitochondrial fractions. See legend to Fig. 1 for method of representing data.

observed by Price, Miller, Miller and Weber (1950). As is shown in Fig. 3, analysis of some of the mitochondrial fractions indicated that the amount of nitrogen was in fact somewhat diminished.

Assays for " free " acid phosphatase activity were, in some instances, performed on supernatant fractions, which showed the same trends as the mitochrondrial fractions except that high values (mean 56% above controls) were found for pre-cancerous liver.

DISCUSSION

Previous studies of enzymic changes within liver cells during azo-dye feeding have been reviewed by Allard (1955). Allard and his collaborators have shown that in the cancerous state, and probably also in the pre-cancerous state, there is a fall in the acid phosphatase activity of mitochondrial fractions. There is also a fall in the Ca^{++}-activated ATP-ase activity, as previously found with transplanted hepatomas by Schneider, Hogeboom and Ross (1950), and by Novikoff, Podber and Ryan (1951) who further showed that this fall was also demonstrable when activation was effected by Mg^{++} as in the present experiments.

" Activation " is not, in the present context, necessarily synonymous with liberation of " latent " ATP-ase activity. Since, however, liberation of this activity does occur in the presence of Ca^{++} (Witter, Watson and Cottone, 1955),

it is likely that the ATP-ase activity measured in these previous studies was equivalent to the " total " activity which was measured in the present experiments, and which showed decreases similar to those reported.

It is uncertain whether the acid-phosphatase activity measured by Allard and collaborators was equivalent to the " total " activity which has now been measured and which, as in the case of the activity measured by these authors, tended to be low in the mitochondrial fractions from cancerous or pre-cancerous liver.

The mitochrondial " free " ATP-ase activity, which is not affected by Mg^{++} (Witter, Watson and Cottone, 1955), and the " free " acid-phosphatase activity showed the expected low values in the control rats now studied. The " free " ATP-ase activity was, however, relatively high with the various liver samples from rats fed azo-dye, as with certain transplanted tumours studied by Potter and Lyle (1951) and by Emmelot and Bos (1955); this increased activity was evidently not merely a consequence of necrosis or of bile-duct proliferation. On the other hand, no such increase was found with " free " acid-phosphatase activity.

The latter activity, as now determined with stored mitochondrial fractions, may be regarded as a measure of the fragility *in vitro* of " lysosomes ", viz. enzyme-containing particles present in mitochondrial fractions but distinct from mitochondria (Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955). If, however, these particles from rats fed azo-dye were already damaged at the time of the mitochondrial centrifugation, acid phosphatase would have become soluble (*cf.* Berthet and Duve, 1951) and the supernatant fraction rather than the mitochondrial fraction would show a high level of " free " activity. The supernatant fraction has in fact shown a high activity after a short period of azo-dye feeding; but the absence of any increase after prolonged feeding suggests that the lysosomes were substantially intact prior to, as well as after, centrifugation and storage of the mitochondrial fraction.

It appears, then, that the administration of carcinogenic azo-dyes, as of certain other dyes or of dinitrophenol (Dianzani and Scuro, 1956), leads to an alteration in mitochondrial properties as judged by the " free " and " total " ATP-ase activities of the isolated mitochondria. Since the azo-dye feeding was usually discontinued several weeks before autopsy, it appears that the alteration is not readily reversed, or is due to an action of the azo-dye (or its metabolites) which can persist for some time.

The nature of this alteration in properties is uncertain. Until recently it was generally considered that a low " free " ATP-ase activity was characteristic of intact mitochondria, and that a high activity signified damage to the mitochondria (Kielley and Kielley, 1951; Kaltenbach and Harman, 1955; cf. Swanson, 1956). It could then be supposed that azo-dye feeding, for as little as 4 weeks, impairs the integrity of the mitochondria *in vivo* or their susceptibility to damage during isolation, in accordance with a hypothesis discussed by Allard (1955).

With the recent application of electron microscopy to the study of isolated mitochondria, it has become evident that " free " ATP-ase is a poor criterion of the morphological integrity of mitochondria (Witter, Watson and Cottone, 1955; Birbeck and Reid, 1956). Mitochondria isolated in the conventional sucrose medium are considerably damaged, whereas mitochondria isolated in certain modified media are apparently little damaged but have " free " ATP-ase activity as high as that of sucrose mitochondria. The interpretation of the present results must therefore await further study with particular reference to the morphological

appearance, under the electron microscope, of mitochondria isolated from liver tissue after azo-dye feeding; the mitochondria in the tissue itself do in fact appear to have undergone pathological changes (Bernhard and Bauer, 1955).

SUMMARY

Mitochondrial fractions have been studied with respect to the changes in the "free" and "total" activity of ATP-ase (assayed in the presence of Mg^{++}) and of acid phosphatase during azo-dye carcinogenesis. The following categories of pathological liver tissue were investigated: precancerous liver; tissue without tumours (from rats fed azo-dye for a prolonged period); mixed tumours (hepatoma and cholangioma); hepatoma with little necrosis; and hepatoma with marked necrosis.

With each category there was found, in comparison with normal liver, a decrease in "total" ATP-ase activity (per mg. of original tissue), but an absolute or relative increase in "free" activity. In the case of acid phosphatase, both "total" and "free" activities tended to decrease; the "free" activity of the supernatant fraction also decreased except in the case of pre-cancerous liver. The interpretation of these findings is discussed.

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